AN ULTRASTRUCTURAL AND MORPHOMETRIC STUDY OF THE ADRENAL CORTEX IN THE INTACT AND HYPOPHYSECTOMIZED MALE RAT

by

Richard Lowell Dickens
B.S., University of Arizona, 1952
D.D.S., Northwestern University, 1956

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ANATOMY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

Approved:

John A. Long
S. L. Wissig
Thomas M. C[illegible]

Committee in Charge

Deposited in the Library, San Francisco Medical Center:

Date .... Librarian

Degree Conferred: JUN 26 1975

XUM

DEDICATION

I dedicate this work to my wife, Junko, for her patience and understanding while enduring the years of deprivation and loneliness.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. John Long for his guidance and words of explanation throughout the course of this investigation. A special debt of gratitude is owed to Yoshiko Hayashino, for without her technical expertise and invaluable assistance this work could not have been accomplished.

XUM

TABLE OF CONTENTS

	PAGE
DEDICATION	i
ACKNOWLEDGEMENTS	ii
INTRODUCTION	1
I. The Purpose of this Study	1
II. The Fine Structure of the Adrenal Cortex of the Intact Rat	3
A. The Zona Glomerulosa	3
B. The Zona Intermedia	6
C. The Zona Fasciculata	7
D. The Zona Reticularis	7
III. The Fine Structure of the Adrenal Cortex of the Hypophysectomized Rat	8
A. The Zona Glomerulosa	8
B. The Zona Intermedia	8
C. The Zona Fasciculata	9
D. The Zona Reticularis	11
IV. Functional Zonation and Control of Secretions of the Adrenal Cortex	11
V. Physiology of the Cortex	14
VI. Previous Quantitative Studies of the Rat Adrenal Gland After Hypophysectomy	16
A. Light Microscopic Studies	16
B. Electron Microscopic Studies	18
VII. Morphometry	20
A. Morphometric Principles	21
B. Random Sampling	23
C. Test Systems	25

TABLE OF CONTENTS (cont'd)

PAGE

MATERIALS AND METHODS 28

I. Preparation of Tissues 28

II. Morphometric Model of the Fasciculata Parenchymal Cell 30

III. Morphometric Procedures 30

A. Stratified Random Sampling 30
B. Systematic Random Sampling 33

IV. Calibration 35

V. Calculation of Absolute Values 35

VI. Statistical Treatment of Data 39

RESULTS 40

I. The Fine Structure of the Zona Fasciculata of the Hypophysectomized Rat 40

II. Morphometry 44

A. Relative Volumes 44
B. Plasma Levels of Corticosterone 46
C. Absolute Volumes 46

III. The Fine Structure of the Zona Intermedia of the Hypophysectomized Rat 48

DISCUSSION 52

I. The Zona Fasciculata of the Rat After Hypophysectomy 52

II. Morphometry 55

III. The Zona Intermedia 63

TABLE OF CONTENTS (cont'd)

PAGE

FIGURES 69

TABLES 108

LITERATURE CITED 111

APPENDIX 121

XUM

INTRODUCTION

I. The Purpose of this Study

That the inner zones of the adrenal cortex atrophy after hypophysectomy has been known for many years (Smith, 1930; Deane and Greep, 1946). Deane and Greep (1946) found that the average cross-sectional area of zona fasciculata cells declined from about 70 μ^2 in the intact animal to about 30 μ^2 in the hypophysectomized animal with most of this change occurring within the first two weeks after pituitary removal. Accompanying this decline in cell size is a coalescence of the lipid droplets which then disappear after several months have elapsed. The zona glomerulosa of the rat does not undergo marked changes after hypophysectomy.

Electron microscopic examination of zona fasciculata cells from intact and hypophysectomized rats has revealed a reduction in the amount of smooth surfaced endoplasmic reticulum and alterations in the size of mitochondria as well as changes in shape of mitochondrial cristae (reviewed by Idelman, 1970).

Attention has been focused on the zona fasciculata because it is the largest zone of the rat's adrenal cortex (deGroot and Fortier, 1959) and it undergoes the most pronounced changes after hypophysectomy (Deane, 1962). At the ultrastructural level, alterations in the size and numbers of lipid droplets, smooth surfaced endoplasmic reticulum and mitochondria have been closely observed because of biochemical evidence that these organelles and inclusions contain cholesterol, the precursor for adrenal steroid hormones, as well as membrane-associated enzymes necessary for the conversion of cholesterol to these hormones (see review by Christensen and Gillim, 1969). It is assumed that alterations in the volume and surface area of these structures will be a reflection of altered secretory activity.

Several electron microscopic studies have been performed with the object of quantitating the ultrastructural alterations which occur after hypophysectomy. Nussdorfer and his co-workers have published a large number of papers which purport to quantitate changes in organellar volume and surface area after various maneuvers designed to stimulate or suppress secretion by the adrenal cortex of the rat (see Nussdorfer et al., 1974 for a list of references to this work). This research has the defect that, while they have determined the volumes of the organelles, they have lumped the intervening cytoplasmic matrix in an unspecified way with one or more of the discrete organelles. Other workers have confined their attention to changes in mitochondrial number and size (Yago et al., 1971; Sekiyama et al., 1971).

None of the workers using quantitative morphologic methods have attempted a correlation of their ultrastructural findings with a functional property of zona fasciculata cells such as secretion of a steroid hormone. It is well known that the principal steroid produced by zona fasciculata cells is corticosterone (Bush, 1953; Giroud et al., 1956). It should be pointed out that both the zona fasciculata and zona reticularis are assumed to produce corticosterone but that the relative importance of the two zones for this process has never been clearly defined for the rat adrenal.

Taking into account the defects in our knowledge outlined above, the objectives of this study can be defined:

1) to make a morphometric study limited to the zona fasciculata of the rat adrenal cortex. This morphometric analysis attempts to correlate alterations in structure of the parenchymal cells with changes in their function, i.e., the secretion of corticosterone, and
2) to investigate and describe the fine structure of the zona intermedia and zona fasciculata as they appear after hypophysectomy.

II. The Fine Structure of the Adrenal Cortex of the Intact Rat

Since some knowledte of the ultrastructure of the rat adrenal cortex is necessary for understanding the changes that take place after the animal is hypophysectomized, a description of the adrenal cortex in intact and hypophysectomized animals will be given.

A. The Zona Glomerulosa

Surrounding the gland itself is a capsule composed of collagenous and elastic fibers, fibroblasts and occasional smooth-muscle fibers.

Directly beneath the capsule is the outermost layer of the cortex, the zona glomerulosa. It is a rather narrow band of cells arranged in loops or arcades. The nuclei are large and oval to round in shape and contain peripheral heterochromatin and a well developed nucleolus.

Certain features are common to the cortical cells of all the zones, but the main distinguishing characteristic is the morphology of the mitochondria (Sabatini et al., 1962; Idelman, 1966). The most conspicuous feature of the cytoplasm of any cortical parenchymal cell is the quantity of mitochondria. This organelle fills every available cytoplasmic space. In the glomerulosa the shape of the mitochondria is usually oval or elongated. The cristae are

XUM

tubular; occasionally, lamellar or vesicular cristae are seen.

I would like to explain the terminology used to describe the appearance of the cristae as seen in electron micrographs. Lamellar crista refers to a plate-like or shelf-like extension from the inner mitochondrial membrane into the matrix of a mitochondrion. This is its appearance when cut in cross section. If the plane of section is parallel with crista then only a blurred line is seen extending across the width of the matrix space.

Tubular crista refers to a hollow, finger-like projection from the inner mitochondrial membrane extending into the matrix. These tubules are usually curved and sometimes follow a tortuous path. The origin of some of the tubules is lost in the plane of section so that these cristae appear to float in the matrix space. Also, if some of the tubules are cut in cross section, they will present the appearance of circular profiles interspersed between the tubules.

Vesicular crista describes a sac-like or spherical crista. These appear as circular profiles suspended in the matrix space. It is not known whether these cristae are free or attached to the inner mitochondrial membrane. Serial sections of a mitochondrion or the use of thick sections observed with the high voltage electron microscope would help clarify this point. Ocassionally, a small tubular neck can be seen budding off from one of these vesicular crista.

Mixtures of the various types of cristae do occur but in the intact animal one type usually predominates.

There is a well developed Golgi apparatus, usually perinuclear in location, consisting of stacks of parallel cisternae with associated vesicles.

XUM

The smooth-surfaced endoplasmic reticulum (SER) is quite prominent, has a tubular shape and fills the spaces between the mitochondria and around the lipid droplets.

The rough-surfaced endoplasmic reticulum (RER) is quite scarce in all cells of the cortex of the rat. Many ribosomes occur free in the cytoplasm which are arranged, for the most part, in clusters.

Microbodies are rarely found. When present, they are usually found as small spherical or ovoid bodies surrounded by a single membrane. Its internal structure is not remarkable in that it seems to be composed of a diffuse amorphous substance of light to moderate density. These structures are also known as peroxisomes because they have been found to contain the enzyme catalase.

There are several structures present in the cytoplasm of adrenal parenchymal cells which I will collectively call "residual bodies" on the recommendation of Long (1973). These lysosomal or lysosome-like bodies are bounded by a single membrane and take a variety of forms. Sometimes they appear as small round multivesicular structures or as very small dark osmiophilic granules. At other times they can be visualized as much larger electron-dense circular objects. Finally, they can contain fine granular material and be of highly variable shape and size at which time they are usually referred to as lipofuscin pigment granules. Residual bodies exist in all the zones of the cortex but increase in number in the deeper zones; the greatest quantity are in the zona reticularis adjacent to the medulla.

Lipid droplets are prominent features of the cytoplasm. There is a moderate amount of lipid in the zona glomerulosa. The lipid droplets are spherical, electron-dense bodies usually surrounded by membranes of the SER, often giving the appearance of being membrane bound. Higher magnification usually reveals, however, that there is no true trilaminar structure of a unit membrane present. Some investigators, nevertheless, believe that lipid droplets are surrounded by a single membrane (Rhodin, 1971; Fujita, 1972).

Glycogen in the form of beta glycogen particles is distributed throughout the cytoplasm but often exists in clumps. Free glycogen particles can be confused with ribosomes but are somewhat larger and more electron dense.

B. The Zona Intermedia

This zone, sometimes referred to as the transitional zone (Deane and Greep, 1946), is found as a narrow band one to four cells thick between the zona glomerulosa and the zona fasciculata. It is a comparatively lipid-free zone and has also been called the sudanophobic zone (Reiss, et al., 1936).

Cater and Lever (1954) have shown that the zona intermedia is not evident in intact rats between 42 and 182 days of age. Since the rats used in this study fall within this age range (51-52 days old), the fine structure of the zona intermedia in the intact animals could not be documented. The following description of the zona intermedia is based on the work of Yoshimura et al., (1968), who used older rats, in order to provide a baseline for my observations on this zone in hypophysectomized animals.

The zona intermedia of the adrenal cortex of the intact rat possesses most of the characteristics of the other zones of the cortex, that is, an abundant supply of SER, very little RER, a well developed Golgi apparatus, many free ribosomes, a small number of residual bodies, and a considerable quantity of glycogen. It must be remembered that the principal distinguishing characteristic of the zona intermedia is its paucity of lipid droplets. Secondarily, the shape of the mitochondria and the type of cristae present within the mitochondria are also the most noteable features of this zone. The mitochondria are large, irregular in shape, and contain long tubular cristae (Yoshimura et al., 1968).

C. The Zona Fasciculata

Just deep to the intermediate zone is the largest zone of the adrenal cortex, the zona fasciculata. The cells of this zone are arranged in parallel cords or strands several cells wide separated by capillaries extending inwards towards the medulla.

Each polygonal cell, which is somewhat larger than its glomerulosal counterpart, contains a large round nucleus with a conspicuous nucleolus. Much of the fine structure is similar to that mentioned above for the glomerulosa but with certain differences. The greatest quantity of lipid appears to be concentrated in this zone and once more the mitochondria provide the most striking basis for distinction. The mitochondria are usually round and contain vesicular cristae (Figures 1 and 2). Some mitochondria are oval or irregular in shape, but the greatest proportion are circular in profile and thus spherical in three dimensions. Occasionally, tubulo-vesicular cristae are observed; nonetheless by far the majority encountered are vesicular.

Because of its size and because of its importance as the major producer of glucocorticoids secreted by the adrenal cortex, the zona fasciculata was selected as the object of study in the present investigation.

D. The Zona Reticularis

The parallel strands of the fasciculata, as they approach the medulla, fuse together to form an irregular network of cells interspersed with capillaries. This, then, is the zona reticularis, the innermost zone of the cortex which borders on the medulla.

The ultrastructure resembles that previously described but with a few variations. The cells are somewhat smaller and there appears to be less lipid present than in fasciculata cells. The mitochondrial appearance is the chief distinguishing feature of this zone. The mitochondria in shape and size resemble those of the fasciculata except the cristae are mainly tubular. At times tubulo-vesicular cristae are discernible. Residual bodies are more numerous in this zone than in any of the more superficial layers of the cortex.

III. The Fine Structure of the Adrenal Cortex of the Hypophysectomized Rat

A. The Zona Glomerulosa

Except for a gradual widening, this zone is largely uneffected by hypophysectomy (Deane and Greep, 1946). This could be predicted by the fact that the glomerulosa is not primarily under the control of ACTH from the pituitary (Deane, 1962).

B. The Zona Intermedia

As is the case with the glomerulosa, the intermediate zone slowly widens with time after hypophysectomy (Lever, 1954). There is little mention in the literature of the ultrastrastructural appearance of this zone following hypophysectomy. Sabatini et al., (1962) report that two weeks after hypophysectomy mitochondria of the intermediate zone tend to resemble those of the glomerulosa of the intact animal. Presumably it was meant that the mitochondria became oval in shape and contained short tubular cristae. Nothing further seems to be published regarding the fine structural appearance of the intermediate zone in the hypophysectomized rat cortex.

C. The Zona Fasciculata

The results of all of the earlier studies done on the fine structure of the zona fasciculata after hypophysectomy are highly questionable because of poor fixation. However, the occurrence of certain trends is of interest. Borowicz (1965) reported a decrease in the quantity of mitochondria while Sabatini et al., (1962) reported a decrease in size of these organelles following hypophysectomy. The latter authors found that 14 days after hypophysectomy, the type of crista present in mitochondria became variable. Some cristae were vesicular, some tubulo-vesicular, and others appeared to be convoluted tubules. Nishikawa et al., (1963) found both a decrease in size as well as a decrease in quantity of mitochondria after hypophysectomy. The quantity of SER was found to decline by Sabatini et al., (1962) whereas Nishikawa et al., (1963) and Borowicz (1965) did not mention changes in the quantity of this organelle. Sabatini et al., (1962) were the only investigators that observed a decrease in the quantity of lipid droplets while Nishikawa et al., (1963) and Borowicz (1965) reported increases in the number of lipid droplets occurring after hypophysectomy.

One of the most recent purely descriptive studies of the fine structure of the adrenal cortex of hypophysectomized rats is that of Fujita (1972). The latest techniques of fixation and embedding were following in the preparation of the tissues so that the quality of the cell preservation appears good from the

XUM

electron micrographs presented. The time periods evaluated were 5, 10, 15, 20, 25, 30, 60 and 90 days after hypophysectomy, with tissues taken from three animals in each group or time period. Ten to 25 days after hypophysectomy, there was a great increase in the number of electron-lucent lipid droplets occurring in most cells of the fasciculata and reticularis. He observed a qualitative difference in these lipid droplets as well since lipid droplets seen in similar cells from intact animals usually appeared somewhat more electron dense. Furthermore, lipid droplets in fasciculata and reticularis cells of intact animals were usually encircled by membranes of the SER. In similar cells from hypophysectomized rats, many droplets were in contact with mitochondria. The mitochondria became quite variable in shape and size and the cristae were almost all converted to the tubular variety. Occasionally, giant mitochondria were encountered also contacting lipid droplets. The quantity of SER was considerably reduced. He noted that the nuclei decreased in size and became irregular in shape. Ninety days postoperatively the pycnotic process continued so that the nuclei were quite shrunken and electron dense. There was also a concomitant reduction in cell size at this time period. The lipid content increased until the cells were completely filled with this substance. Heterogeneous dense bodies (residual bodies) occupied much of the cytoplasm. In the extracellular space he observed an unusual accumulation of collagenous fibrils, macrophages and degenerating cortical cells.

XUM

Luse (1967) reported on male rats examined two years following hypophysectomy. Adrenocortical cells were found to contain a great quantity of residual bodies but were totally devoid of lipid droplets. The cortical tissue was heavily infiltrated with plasma cells and lymphocytes. It is evident that the lipid completely disappears after long-term hypophysectomy.

D. The Zona Reticularis

Very little seems to have been published concerning the ultrastructural appearance of the reticularis after hypophysectomy. One probable reason is that the distinction between the fasciculata and reticularis based on mitochondrial differences becomes nonexistent about 10 days following hypophysectomy (present observations). The authors of two of the publications mentioned above (Sabatini et al., 1962 and Fujita, 1972) referred to the fasciculata and reticularis as a single entity when describing changes observed after hypophysectomy.

IV. Functional Zonation and Control of Secretions of the Adrenal Cortex

It is generally conceded by most investigators in the field that the histologic zonation observed in the adrenal cortex is accompanied by a functional zonation. This is to say, each zone in the cortex secretes certain hormones that have specific functions. This theory proposes that the inner zones of the fasciculata and reticularis are under control of ACTH from the adenohypophysis. ACTH stimulates the secretion of glucocorticoids from the inner zones while the outer zona glomerulosa, which is relatively free of pituitary control, secretes mineralocorticoids.

This functional zonation theory was first advanced by Swann in 1940 and since that time has gained considerable experimental support. More specifically, in reference to the rat, the zona glomerulosa secretes aldosterone and zonae fasciculata and reticularis produce corticosterone. Giroud et al. (1956) and Sheppard et al (1963) have provided evidence for the zonation theory in the rat. It is possible to strip off the capsule of the adrenal gland separating the gland into capsular and decapsulated portions. Histologic examination of the capsular portion has shown it to be composed of fibrous tissue and zona glomerulosa cells as well as a small number of zona fasciculata cells. Giroud et al. (1956) incubated both the capsular and decapsulated portions of the rat adrenal gland in precursors of steroid hormones and demonstrated that almost all of the aldosterone was produced by the capsular portion of the gland while the decapsulated portion (containing the zonae fasciculata, reticularis and medulla) produced mostly corticosterone. In 1963, Sheppard et al. repeated the above experiment utilizing capsular and decapsulated rat adrenal glands but incubated in the presence or absence of ACTH to demonstrate that ACTH has little effect on the capsular portion of the gland but does stimulate the secretion of corticosterone by the decapsulated portion of the gland.

While not part of the theory as originally proposed by Swann, it is now generally thought that androgens are primarily synthesized in the reticularis and to a lesser degree in the fasciculata (Jones and Griffiths, 1968). It is also believed that estrogens may be produced by the cells of the two inner zones but experimental evidence for this concept is meager. For a more detailed account of this subject, see the review by Griffiths and Cameron (1970).

As mentioned, the zona glomerulosa is relatively independent of ACTH control since the zona glomerulosa does not atrophy and the animal does not lose sodium when the pituitary is removed (Dean and Greep, 1946; Deane et al., 1948). Control of the production of aldosterone is now thought to be under the influence of the complex renin-angiotensin system (see review by Ganong and Van Brunt, 1968).

ACTH is necessary for both growth and steroidogenesis of the rat adrenal gland. In vivo studies have shown that there are pronounced increases in adrenal weight, protein and nucleic acid content of the rat adrenal after prolonged administration of ACTH (Farese, 1968). In vitro studies have demonstrated that ACTH enhances steroid output from cells derived from the decapsulated portion of the rat adrenal gland (Sheppard et al., 1963).

Following hypophysectomy, there is a dramatic decrease in the weight of the adrenal gland (Smith, 1930; Deane and Greep, 1946) and a similar decline in glucocorticoid secretion (Deane, 1962). Microscopic examination of the gland reveals atrophy of the two inner zones while the zona glomerulosa remains relatively unaffected. If hypophysectomized animals are then given injections of ACTH, the atrophic effects are reversed, and the gland returns to its normal morphologic appearance and functional activity (Deane, 1962). Prolonged administration of ACTH to an intact animal causes hypertrophy of the fasciculata and reticularis with increased glucocorticoid output (Deane, 1962). If an intact animal such as the rat is given long-term injections of a potent glucocorticoid such as corticosterone, effects similar to that of hypophysectomy are produced in the adrenal cortex, i.e., loss of adrenal weight and atrophy of the two inner cortical zones (Deane, 1962).

Accumulated evidence has led to the present concept of how the inner zones of the cortex are controlled. Corticotropin releasing factor (CRF) is produced by certain neurons of the hypothalamus. These neurons are sensitive to the circulating blood levels of glucocorticoids. Increased glucocorticoid levels produces decreased secretion of CRF and vice versa. Axons from these neurons end on portal blood vessels in the median eminence. This portal system carries CRF to the adenohypophysis where it stimulates the production and release of ACTH (Ganong, 1963; Mess and Martini, 1968).

V. Physiology of the Cortex

Although many steroid hormones are elaborated by the adrenal cortex, only those pertaining to the rat will be mentioned in this brief account of adrenal cortical physiology.

The principal mineralocorticoid secreted by the zona glomerulosa is aldosterone. Aldosterone is responsible for sodium resorption by the distal convoluted tubule of the kidney and the conservation of sodium by resorbing sodium ions from sweat, saliva and intestinal secretions, thus aiding in water retention for maintaining normal blood pressure and volume.

Both the fasciculata and reticularis secrete corticosterone and androgens, but the fasciculata secretes primarily corticosterone while the reticularis is mostly concerned with the production of androgens (Long, 1974). Corticosterone, the principal glucocorticoid secreted by the rat's adrenal cortex (Bush, 1953 and Moses et al. 1969), has many effects, but for the most part

XUM

it increases the rate of gluconeogenesis, increases glycogen storage by the liver, and produces an anti-insulin effect by creating a hyperglycemia. It also causes an elevation in circulating free fatty acids released from adipose tissue and is necessary for the animal's resistance to a variety of stresses (Ashmore and Morgan, 1967).

Stimulation of the secretory activity of the cortex causes the parenchymal cell to enlarge. Acute, severe stimulation can cause total loss of lipid droplets as can a severe, chronic stimulation. Chronic, moderate stimulation brings about an increase in quantity but a decrease in size of lipid droplets (Deane, 1962). Conversely, decreased secretory activity of the inner two zones, as occurs after hypophysectomy, results in cortical cell atrophy. The lipid droplets apparently coalesce and their numbers increase, at least up to three months after pituitary removal (Fujita, 1972). Hypophysectomy for longer periods brings about a total depletion of lipid from cortical parenchymal cells (Luse, 1967). It is evident, therefore, that the quantity of lipid and the size of cortical cells varies with physiologic activity.

Figure 3 is a diagram of the biosynthetic pathways of the principal corticosteroids. All of the enzymes responsible for this synthesis are associated with the membranes of mitochondria or endoplasmic reticulum (microsomes). Christensen and Gillim (1969) provide a detailed review of the evidence for assignment of enzymatic activity to each of the subcellular components.

One of the main assumptions made in quantitative morphology is that changes in the volume, number or surface area of an organelle will reflect synthetic activity. Put another way, changes in surface area or quantity of a membranous organelle will provide more or less area for the insertion of enzymatic proteins and will thus determine the synthetic activity of the cell.

VI. Previous Quantitative Studies of the Rat Adrenal Gland After Hypophysectomy

A. Light microscopic Studies

1. One of the first reports on the effects of total ablation of the pituitary gland on the adrenals of the rat was that of Smith in 1930. Smith also pioneered the paraphrayngeal approach to hypophysectomy which led to uniformly consistent total ablation of the pituitary. Prior to this time, hypophysectomy via the temporal approach utilizing chromic acid had been employed. Often the later method resulted in only partial destruction of the hypophysis or damage to the brain or both leading to conflicing reports as to the symptoms produced.

Smith found that the combined weight of the adrenals was reduced by some 60 to 89% in male rats autopsied fifteen days or more after hypophysectomy. In a further step, camera lucida tracings of serially sectioned glands from both intact and hypophysectomized animals were made. Planimetric studies were performed on these sections to determine the percentage of cortex and medulla in each gland. For intact rats the cortex occupied about 91% and the medulla about 9% of the total volume of the gland. Corresponding figures for the hypophysectomized rats were about 75% cortex and 25% medulla. The results demonstrated that the glandular atrophy was confined to the cortex with the medulla being relatively uneffected. He stated that this atrophy was not due to loss of cells but to a decrease in cytoplasmic volume.

2. In their classic paper of 1946, Deane and Greep repeated the quantitative studies of Smith, Male rats were studied 2, 4, 6, 8, 10, 14 28, 56 and 136 days after pituitary removal. They found that the weight of the paired adrenal glands had declined by about 74% at the end of the 136 day periods as compared to intact controls; more than 50% of this loss occurred in the first 14 days. Using camera lucida tracings of the largest section of a gland, the areas of the cortex and medulla were determined planimetrically. The tracings indicated that the adrenal weight loss was due to shrinkage of the cortex. This decrease in cortical area amounted to about 50%. All of the decrement occurred in the first 14 days after hypophysectomy. A third experiment was conducted to learn if this decrease in cortical area was the result of cellular atrophy or loss of cells or both. All of the cells in a standard-width segment of the cortex were counted from camera lucida tracings of hematoxylin-stained preparations. The area of this standard-width segment divided by the number of cells contained within it yielded the average area of one of the cells. The results indicated that the average area occupied by cells declined by 50% in the first two weeks, and virtually no change occurred after that time. The total number of cells in the standard segment remained constant for at least one month, thus supporting the conclusions of Smith made 16 years earlier that

cortical atrophy was due to shrinkage of cells rather than to decrease in number of cells.

B. Electron Microscopic Studies

Several groups of investigators have applied stereologic methods to adrenocortical cells of intact and experimentally treated rats. A uniform method of presenting morphometric results has not evolved making comparisons of data from various investigators either difficult or impossible. It is possible to discern trends which is all that will be emphasized in that which follows.

Nussdorfer and his co-workers have reported a decline in cell volume of about 40% ten days after hypophysectomy (Nussdorfer and Mazzocchi, 1972; Nussdorfer et al., 1973), whereas prednisolone treatment of intact rats induces a 52% decrease in cell volume in 4 days (Nussdorfer, 1970b). Although the initial weights and ages of the animals used in these experiments are approximately the same, this group of workers reports the absolute volume of fasciculata cells from intact animals as ranging from 1,545 μ^3 (Nussdorfer, 1970b) to 1,878 μ^3 (Nussdorfer et al., 1973). This is an 18% variation in the control values obtained by one laboratory.

Lipid inclusions are usually reported to increase in total quantity after hypophysectomy (Deane, 1962) although the information in this reference is non-quantitative. Canick and Purvis (1972) find a progressive increase in total volume of lipid per fasciculata cell up to 14 days after hypophysectomy. By contrast, the Nussdorfer group reports a 5% (Nussdorfer and Mazzocchi, 1972) or a 28% (Nussdorfer et al., 1973) decrease in lipid volume 10 days after hypophysectomy. Their control values differ by 47% in these two experiments.

Relative volume of mitochondria in intact rats (the fraction of the total fasciculata cell volume occupied by mitochondria) show a wide variation in the values reported by several investigators. These values range from 28.4% (Nussdorfer, 1970b) to 41.9% (Nussdorfer and Mazzocchi, 1972) with a very high value of 47.5% being given by Magalhães and Magalhães (1969). Sekiyama et al. (1971) observed a small increase in mitochondrial relative volume three days after hypophysectomy, and Nussdorfer et al. (1973) also find an increase in relative volume of mitochondria although the absolute volume per cell of this organelle exhibits a 50% decrease 10 days after hypophysectomy.

The endoplasmic reticulum is found to decrease in absolute volume by 40-50% during the 10 days following hypophysectomy (Nussdorfer and Mazzocchi, 1972; Nussdorfer et al., 1973).

In all of the work reported by Nussdorfer and his co-workers, no mention is made of the portion of the cell I am calling cytoplasmic matrix, that is, the space intervening between the discrete organelles. Nussdorfer may have lumped this compartment with some other organelle, but since his papers are silent on this point, the data from Nussdorfer's laboratory cannot be meaningfully compared to my data or to that of other workers. In addition, other signs of carelessness are evident in his papers. A particularly bad example is his paper of 1970 wherein the sum of the volumes of the individual components exceeds the reported total cell volume by 10%.

It is evident that wide divergences in normal values exist and that further information is desirable.

VII. Morphometry

With the advent of morphometric techniques for quantitative analysis of internal cell structure on electron micrographs of tissue sections, a new dimension of investigation has been created for the cell biologist. These techniques were first applied to biological tissues by Loud (1962) and Weibel (1963). The major credit must go to Weibel and his associates for developing practical morphometric methods for use in any electron microscopic laboratory (Weibel et al., 1966; Weibel, 1969; Weibel and Bolender, 1973).

Morphometry and stereology are two terms that are commonly employed interchangeably, but according to Weibel (1969) they are not synonymous. Weibel states that morphometry suggests the use of any quantitative method for describing structure, whereas stereology implies a geometric analysis of profiles of two-dimensional structures to yield three-dimensional information about those structures. Using these definitions, stereology is the more appropriate term for the present investigation, but, regardless of which is used, the stereological method is an important research tool. Not only can structure be correlated with function by the use of this method, but it is an effective procedure that can detect the presence of structural changes that take place within cells. Furthermore, these changes can be subjected to statistical analysis to determine if these changes are indeed significant.

XUM

A. Morphometric Principles

The principle upon which morphometric analysis is based is that quantitative information about the composition in three dimensions of tissue can be derived from measurements taken from a two-dimensional representation of that tissue, which in the present case would be a photograph of a slice of tissue. This relationship between the two and the three-dimensional properties is only true in a statistical sense in that a large number of measurements of two-dimensional profiles must be made to obtain average dimensions. In a micrograph, then, cross-sectional views of components depict volumes as areas, surfaces as lines, and lines as points. The usual method of measuring these various structures on a micrograph is to superimpose a test system of points or lines and to measure the number or length of these points or lines that fall on the component under study. The fundamental principle underlying this method is that of Delesse (1848) which states that the fractional volume occupied by the component i is equal to its fractional area in a random cross section. This principle has been further extended by Rosiwall (1898) and Glagoleff (1933) so that the fractional volume is also equal to the fractional length of random penetrating lines or the fraction of random points contained within it. Using the notation of Weibel (1969) this can be summarized:

$$V_{V_i} = A_{A_i} = L_{L_i} = P_{P_i}$$

This notation requires some explanation. The first capital letter refers to the parameter and the second capital letter, written as a subscript, identifies the reference system. Lower case letters identify the component to which the parameter refers. Therefore, V_{V_i} is the volume fraction occupied by

XUM

the component i, A_{A_i} is the area fraction of the section occupied by i, L_{L_i} is the fraction of the test line intercepting i and P_{P_i} is the fraction of test points enclosed in i.

We now have three methods of determining volume fraction V_{V_i}: (1) by measuring the area fraction of the component i through use of laborious planimetry, (2) by determining the lineal fraction of test lines running through the component i, or (3) by superimposing a regular point lattice on a micrograph and counting the points falling on i. The latter is the most efficient method because the measurement necessary is simply a counting procedure. Hennig (1959) also demonstrated that point counting procedures are for statistical reasons preferable to lineal integration. For these reasons, point counting procedures were used exclusively in the present investigation for volume fraction determinations.

Since the Delesse principle is so basic to an understanding of morphometric reasoning, a simple demonstration of the rationale behind it seems in order.

A thin section of tissue prepared for electron microscopy is about 600-800 Å thick. For all practical purposes, all the components contained within this ssection have the same thickness, t, as the section itself. By adding all the cross sectional areas of the component i together, A_i, and multiplying by t, the partial volume $V_i = A_i t$ of the component contained in the section is obtained. The total surface area, S, of the section times t gives the total volume of the section $V_S = A_S t$. The volume fraction of the component i is

$$V_{V_i} = \frac{V_i}{V_s} = \frac{A_i t}{A_S t} = \frac{A_i}{A_S}.$$

since the layer thickness t cancels out. Formal mathematical proof of the Delesse principle appears many times in the literature. Two of the more recent discussions are those of Weibel (1963), and Weibel and Bolender (1973).

Membranes such as those of the SER appear in cross section as lines. The length of these lines found per unit area of micrograph is proportional to the area of the membranes per unit volume (Tomkeieff, 1945). The length of membrane profile can be measured by superimposing a test system of lines and counting the intersections of this profile with these lines. The area of membrane included in a unit test volume is called the surface density, S_{V_i} (Weibel et al., 1966),

$$S_{V_i} = \frac{2 I_i}{L_T} = 2 I_L$$

where I_i is the number of intersections of membrane with lines of total length L_T and I_L is the number of membrane intersections per unit length of sampling line. Other parameters are available for morphometric analysis (see below) but relative volume and surface density are the two most pertinent to the present study.

B. Random Sampling

Morphometric measurements are based on statistical and geometric principles. They originate from the probability with which profiles of structures coincide with a suitable test system. Therefore, it is necessary that the tissue sample and test system coincide in a bias-free random process. This means that strict random sampling procedures must be adhered to at all

XUM

stages of the process from selection of tissue blocks to recording and analyzing the electron micrographs.

The sample also should be representative of the tissue being studied. For this reason, the electron micrographs must be thoroughly dispersed throughout the entire organ or gland under investigation. Adequate sample dispersion can be achieved by simple or chance random sampling, systematic random sampling, or by stratified random sampling or by any combination of these procedures. In the case of systematic random sampling, the dispersion of samples is made certain by their regular spacing within a sampling lattice. Although on the surface this procedure seems nonrandom, the sampling is still random as long as certain conditions are met. (1) The lattice is randomly applied to the tissue, and (2) the tissue is not composed of individual units preferentially oriented with respect to the plane of section. Fortunately, most glandular tissue fulfills the latter condition by having their functional units randomly dispersed throughout the tissue (Weibel and Bolender, 1973). Ebbesson and Tang (1967) have compared the three random sampling procedures discussed and found that systematic random sampling gave the smallest standard error, simple random sampling gave the largest standard error with stratified random sampling standing midway between the two.

In the present study, stratified random sampling and systematic random sampling procedures were utilized.

XUM

C. Test Systems

The test systems that are used for morphometric analysis usually take the form of regular lattices of points and lines. Several of these test systems are illustrated in Figure 4. The top two (A and B) are multipurpose test systems (Weibel et al., 1966) because they combine points (located at each end of the short line Z) for estimating volumes, lines for estimating surface densities, and areas for estimating the length of curved features and for particle counting. The bottom test system (C) is a double-lattice test system which is useful for the estimation of the volume densities of scarce components. A scarce component is defined by Weibel (1969) as one occupying 5% or less of the cytoplasmic volume. Each point on the test systems represents an area. For the multipurpose test system composed of short test lines of length Z whose end points are arranged in a regular triangular lattice, the lattice unit is not a square but rather an equilateral rhombus of area $a = Z^2 \frac{\sqrt{3}}{2}$. Each point on this system is equal to the area $a = Z^2 \frac{\sqrt{3}}{2}$. For the double-lattice test system, the lattice unit is the small square c^2 so that each point represents the area $a = c^2$. Therefore, it can be seen that each point in either of these test systems is equal to an area, and this area is equal to a volume by Glagoleff's principle (1933) as previously discussed. The composition of the double-lattice test system is arranged so that every g-th line is drawn heavier than the others.

Estimation of the relative volume V_{V_i} of the scarce component i is quite efficient because it only requires counting the number of fine cross-points P_i

XUM

falling on i and the number of coarse cross-points P_i' falling on cytoplasm to yield V_{V_i} by the formula

$$V_{V_i} = \frac{P_i}{g^2 P_i'}$$

The construction of the test systems depends on several factors. In essence it can be said that the optimal density of points depends on the size and frequency of the component under investigation. Thus, it is necessary to make preliminary measurements or counts of the components under investigation for the particular tissue being studied to determine these size and frequency factors. In the case of the double-lattice test system for studying scarce components, at least one point should fall on every profile of the component to prevent underestimation. The distance should be chosen so that c^2 is similar to or somewhat smaller than the mean profile area of that scarce component. Similarly, in the case of the multipurpose test system, the area $a = Z^2 \frac{\sqrt{3}}{2}$ should be selected so that it is somewhat larger than the maximal profile area of the structure of interest in order that no more than one point can fall on the same profile. For further details on this point, the reader is referred to the treatises of Weibel (1969), and Weibel and Bolender (1973).

For the purpose of estimating volume densities, it is important to know the total number of points that need to be counted in order to keep the relative error to a certain level. The relationship between relative error in volume density estimation E_{V_V} and test point number P_T is shown in a graph (Figure 5). In actual practice, the volume density of a component

XUM

is roughly estimated on a few micrographs. After selecting an acceptable relative error, the test point number is read from the abscissa.

As an example from my work, a preliminary mitochondrial count of the intact control tissue revealed that the mitochondria occupied about 40% of the cytoplasmic volume. In order to keep the volume density relative error below 5% some 500 points had to be counted. On the other hand, preliminary estimation revealed that the lipid volume was a scarce component at less than 5%. It can be seen from the graph that over 3500 points had to be counted to keep the relative error below 5%. Of course, many more points than this were actually counted. As a matter of fact, in the present investigation the volume density relative error estimations were kept below 5% except in a few instances where a less than 10% relative error value was accepted.

The information that can be gained from the use of stereological techniques for the study of tissue or cell structure resides mostly in the following few parameters:

1. relative volume or volume density or volume fraction (all are synonymous).
2. surface density, i.e., the surface area of a component per unit volume.
3. numerical density, i.e. the number of particles per unit volume.
4. mean diameter of structures.

MATERIALS AND METHODS

I. Preparation of Tissues

51 or 52 day old male Long-Evans rats weighing 164-240 g were used in this study. A total of 30 rats divided into 6 groups - 5 rats in each group was studied. The 6 groups investigated were;

1. intact controls
2. 1 day after hypophysectomy ($\overline{H}$)
3. 2 days after $\overline{H}$
4. 4 days after $\overline{H}$
5. 10 days after $\overline{H}$
6. 14 days after $\overline{H}$

Five groups of animals were hypophysectomized by the paraphrayngeal approach. Evidence for the completeness of the operation was the cessation of growth, body weight loss, and, at autopsy, the decrease in adrenal size as well as direct visual inspection of the sella turcica.

The animals were sacrified by decapitation between 9:00 - 10:00 a.m. Blood was collected from all animals. This was centrifuged and the plasma was frozen and stored at -5°C for subsequent corticosterone determinations (see Appendix for method). After laparotomy, the left adrenal was taken and the fat removed from the capsule of the gland. Each gland was cut in half and half was placed in Bouin's fixative for light microscopy. The other half, for electron microscopic use, was diced into tissue blocks approximately 1 mm on each side and immersed in cold glutaraldehyde-H_2O_2 fixative (Peracchia and Mittler, 1972) prepared as follows:

50% glutaraldehyde (Fisher Scientific Co.) was diluted to 3% with 0.1M sodium cacodylate buffer at pH 7.4. Just before tissue immersion, 2 drops of 30% H_20_2 (Mallinckrodt Chemical Works) were added to each ml of 3% glutaraldehyde and the mixture thoroughly shaken. Fixation was for 2 hours in this solution and then the tissues were changed to a cold 3% glutaraldehyde solution prepared as follows: 8% glutaraldehyde (Polysciences, Inc.) was diluted to a 3% solution with 0.2M sodium cacodylate buffer at pH 7.4. Fixation continued in this solution for 1 hour. The tissues were washed for 1 hour in 0.1M sodium cacodylate buffer containing 7% sucrose. Two buffer changes were made and the tissues were stored overnight at 4°C in the buffer. The tissues were post-fixed in 2% $0s0_4$ buffered with sodium veronal acetate to pH 7.4 for 1 hour (Palade, 1952). They were then dehydrated in a series of acetone solutions and embedded in Epon 812 (Luft, 1961). 0.5 μm sections stained with toluidine blue were examined in the light microscope for tissue orientation. For electron microscopy, silver to gold sections, cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome, were collected on copper grids of 200 or 400 mesh coated with a carbon-stabilized parlodion film. One section was placed on each copper grid so that each block provided only one sample for morphometric evaluation. The sections were stained with a 5% uranyl acetate solution for 30-45 minutes at 45-60°C. They were then stained with a 0.4% lead citrate solution in an oxygen atmosphere for 4-5 minutes (Venable and Coggeshall, 1965). The sections were examined in a Hitachi HS-8 electron microscope at an accelerating voltage of 50 KV.

II. Morphometric Model of the Fasciculata Parenchymal Cell

Figure 6 is a diagrammatic representation of the model of the fasciculata parenchymal cell. It can be seen that the cell is divided into its two major compartments of nucleus and cytoplasm. The cytoplasmic compartment is further subdivided into cytoplasmic matrix, mitochondria, lipid and endoplasmic reticulum (ER). For the present study, the cytoplasmic matrix is defined as being composed of ground substance, residual bodies and microbodies, which is to say everything in the cytoplasm except the mitochondria, lipid and ER. By the same token, the ER is defined as including the rough-surfaced endoplasmic reticulum (RER), the smooth surfaced endoplasmic reticulum (SER), and Golgi membranes. The Golgi complex is small in adrenocortical cells and its boundaries are difficult to define. The quantity of this organelle does not appear to undergo major changes after hypophysectomy and therefore, Golgi membranes are included in the ER for the purposes of this study. Also given in the diagram are the symbols used for the morphometric parameters in terms of volume densities or surface density. Surface density measurements were obtained only for the ER compartment (SVer). All absolute values in this study refer to the cytoplasm of an average fasciculata parenchymal cell. Relative values, except for nuclear volume, are also given in relation to the cytoplasmic volume of this average cell. With the use of this model it is possible to account for the changes occurring within the cell due to experimental manipulation.

III. Morphometric Procedures

A. Stratified Random Sampling

For each rat in each of the time periods, 10 Epon blocks were made from pieces of tissue taken from throughout the cortex of the gland. From these 10 blocks, 5 were randomly selected for thick sectioning. This made a total of 25 blocks sectioned for the 5 rats in each of the time periods up to the 10th day after hypophysectomy ($\overline{H}$) period. For the 10 and 14 day $\overline{H}$ rats, 3 blocks were randomly selected making a total of 15 blocks sectioned for each of the last two groups of animals. In all there were 130 blocks sectioned for the entire investigation.

In order to properly analyze the tissues, 3 levels of sampling were chosen (Figure 6).

Level I

Light micrographs were taken at an initial magnification of 200X and enlarged to a final print magnification of 1500X. This level of magnification was adequate to determine the volume fraction occupied by nuclei and cytoplasm of zona fasciculata parenchymal cells. The table below shows the number of photomicrographs taken for the various time periods.

Time Periods	Photomicrographs
Intact Controls	63
1 day after $\overline{H}$	74
2 days after $\overline{H}$	76
4 days after $\overline{H}$	75
10 days after $\overline{H}$	46
14 days after $\overline{H}$	48
Total	382

XUM

Level II

Electron micrographs were taken at an initial magnification of 6400X and enlarged to a final magnification of about 16,500X. This magnification was used for determining relative cytoplasmic volumes of lipid and mitochondria. The table below shows the number of photomicrographs taken for the various time periods.

Time Periods	Photomicrographs
Intact Controls	234
1 day after $\overline{H}$	131
2 days after $\overline{H}$	126
4 days after $\overline{H}$	131
10 days after $\overline{H}$	87
14 days after $\overline{H}$	80
Total	789

Level III

Electron micrographs were taken at an initial magnification of 20,000X and enlarged to a final magnification of about 66,000X. This magnification was deemed adequate for determining the relative cytoplasmic volume of the endoplasmic reticulum and its surface density. The table below shows the number of photomicrographs taken for each time period.

Time Periods	Photomicrographs
Intact Controls	117
1 day after $\overline{H}$	139
2 days after $\overline{H}$	182
4 days after $\overline{H}$	139
10 days after $\overline{H}$	95
14 days after $\overline{H}$	90
Total	762

At levels II and III fields containing less than 50% cytoplasm were discarded because at these levels all values were to be estimated with respect to the cytoplasm.

B. Systematic Random Sampling

In order to insure randomness in the recording of the electron micrographs, a method for preventing bias in the selection of the fields to be photographed must be adopted. A method should be used that allows the position of the micrographs to be fixed with respect to a reference system that is independent of the structures being photographed. A simple way of accomplishing this is advocated by Weibel et al. (1966) and was the procedure that was followed. The specimen grid bearing only one tissue section was positioned so that the upper left corner of the grid opening coincided with the upper left corner of the viewing screen; the area was photographed and the specimen stage was moved to the next square of the grid where the procedure was repeated. The micrographs which were thus secured were analyzed by placing appropriate

XUM

test systems over them and the necessary number of hits and cuts of points and lines with the various component profiles were recorded on a blood cell counter. The raw data was put into the proper formulas to obtain the desired information. For relative volume V_{V_i} the formula is:

$$V_{V_i} = \frac{P_i}{P_T}$$

where P_i is the number of points contained within the profile of the component i and P_T is the total number of points falling on the section. For surface density S_{V_i} the formula is:

$$S_{V_i} = 2\frac{I_i}{L_T}$$

where I_i is the number of intersections of membrane with the test lines of total length L_T.

The test systems used took several forms. At Levels I and II, two different test systems were employed. The first was a multipurpose test system containing 84 lines and 168 points which measured 24 x 24 cm with a point spacing of 1.5 cm. This test system was used to estimate the relative volumes of nucleus and cytoplasm at Level I and of mitochondria and lipid at Level II. A double-lattice test system was utilized for estimating the relative volume of lipid for intact animals only, as it was found that at this time period the lipid was a scarce component. This system had a 1:9 ratio of coarse to fine lines and a coarse line separation of 1.5 cm and fine line separation of 0.5 cm. At Level III another multipurpose test system similar to the first one was used but this system measured 27 x 27 cm with a point spacing of 2.0 cm. Estimations of the volume and surface densities of the ER were obtained with the latter test system.

IV. Calibration

For light microscopy, the microscope was calibrated with a scale graduated in 0.01 mm to determine final print magnification. To establish the final magnification for electron microscopy, a photomicrograph was taken of a magnification standard at the end of each photographic session. The standard used was a carbon replica of a diffraction grating having a spacing of 4629 Å.

V. Calculation of Absolute Values

Nuclear volume computations were based on the fact that rat fasciculata nuclei are essentially spherical in shape (Palkovits and Fischer, 1968). One hundred fasciculata nuclei were measured on 0.5 μm thick tissue sections stained with toluidine blue for each of the time periods using a Zeiss micrometer eyepiece in a light microscope. For the 10 and 14 day $\overline{H}$ animals, when the nuclei are no longer spherical, the mean of a length and width measurement for each nucleus was obtained. The mean nuclear diameter at each time period was used to calculate nuclear volume assuming the nucleus is a sphere $V = \frac{\pi d^3}{6}$. I realize that there are more sophisticated methods for calculating nuclear volume but this one seemed adequate for the limited time and facilities that were available.

Absolute cytoplasmic volume was determined by using the relative volume data obtained from point counting procedures in combination with the absolute nuclear volume data through application of the equation:

$$\frac{\text{absolute nuclear vol.}}{\text{relative nuclear vol.}} = \frac{\text{absolute cytoplasmic vol.}}{\text{relative cytoplasmic vol.}}$$

and solving for absolute volume.

Basic to all the absolute value conversions relating to cytoplasmic or cell volumes is the calculation of absolute nuclear volume by any of a number of methods such as the one used in this study. Once this value is obtained it is a relatively simple matter to convert all relative values to absolute values by the method given below.

First, one must multiply absolute cytoplasmic or absolute cell volumes, which were directly calculated from absolute nuclear volume, by the relative volumes of the components as determined by point counting procedures to give absolute volumes of the components in terms of cytoplasmic volume or cell volume respectively. For example, data from intact control animal number one yielded the following relative volumes of lipid (1), mitochondria (m), endoplasmic reticulum (er) and cytoplasmic matrix (cm); $V_1 = 0.028$, $V_m = 0.113$ and $V_{er}=0.113$ $V_{cm} = 0.436$. Multiplying these values by the absolute cytoplasmic volume for animal number one ($2,443.0\ \mu m^3$) gives absolute volumes of these organelles in terms of cytoplasmic volume.

$V_1 = 0.028 \times 2,443.0 = 68.4\ \mu m^3$/cell cytoplasm

$V_m = 0.423 \times 2,443.0 = 1033.4\ \mu m^3$/cell cytoplasm

$V_{er} = 0.113 \times 2,443.0 = 276.1\ \mu m^3$/cell cytoplasm

$V_{cm} = 0.436 \times 2,443.0 = 1065.1\ \mu m^3$/cell cytoplasm

The relative volume of a component expressed as a fraction of the total cell volume is calculated by dividing the absolute volume of the component per cell cytoplasm by the total cell volume. For control animal number one the absolute cell volume is $2,667.3\ \mu m^3$.

XUM

$$V_1 = \frac{68.4\ \mu m^3}{2667.3\ \mu m^3} = 0.026$$

$$V_m = \frac{1033.4\ \mu m^3}{2667.3\ \mu m^3} = 0.387$$

$$V_{er} = \frac{276.1\ \mu m^3}{2667.3\ \mu m^3} = 0.104$$

$$V_{cm} = \frac{1065.1\ \mu m^3}{2667.3\ \mu m^3} = 0.399$$

$$V_n = \frac{244.3\ \mu m^3}{2667.3\ \mu m^3} = 0.084$$

Values for the other four animals in the intact control group were obtained in the same way. A mean was calculated for all five animals and this absolute value was used to make up Table 3. In a similar fashion, the absolute values for the other groups were calculated so that the values for all the parameters listed in Table 3 represent a mean from five animals.

Calculation of surface density, S_{Ver}, for the endoplasmic reticulum (ER) was determined by the following formula:

$$S_{Ver} = \frac{2 \cdot ler}{L_T}$$

when ler is the number of intersections of the test line with the surface contour of the ER and L_T is the length of the test line. The total surface area of ER in the cytoplasmic volume was obtained by multiplying these data by the absolute cytoplasmic volume for the various time periods to yield the product

XUM

in μm^2. L_T was determined by first obtaining the unmagnified length (Z) of the short line probe. This was done by dividing its actual length by the final print magnification. L_T was then found by using the formula:

$$L_T = \tfrac{1}{2}P_T \cdot Z \cdot M$$

where P_T is the number of test points and M is the number of micrographs on which the test system was used. As an example, data from intact animal number one will be used. The actual length of Z on the multipurpose test system was 2.0 cm. The final print magnification for the micrographs at Level III was 66,877X. After convering cm to μm, $Z = \frac{20,000}{66,877} = 0.3\ \mu m$, the unmagnified length of Z. The number of points on the test system was 168. Therefore, $L_T = \frac{1}{2} \times 168 \times 0.3 = 25.2\ \mu m$. Ier which was determined by intersection counting was 2512. The total number of micrographs sampled was 29 and the absolute cytoplasmic volume was $2249.6\ \mu m^3$. These values were then placed in the surface density formula to yield:

$$S_{Ver} = \frac{2 \times 2512}{29 \times 25.2} \times 2249.6 = 15,465.2\ \mu m^2$$

It is sometimes desirable to obtain the absolute volume of parameters in relation to the body weight of an animal. This can be accomplished if the volume of the gland or organ is known by merely multiplying the absolute volume of gland by relative volume of component. A relatively simple method for determining glandular volume is that of Scherle (1970). The weight (in grams) of the gland in air minus its weight in liquid is equal to its volume (in cm^3), provided the specific gravity of the liquid is close to that of water (~ 1.0). This

XUM

relationship can be understood by inspecting the following formula:

$$V_o = \frac{W_A - W_L}{SG_L}$$

where W_A is the weight of the organ in air, W_L is its weight in liquid, and SG_L is the specific gravity of the liquid. However, it was found during the course of this investigation that the results obtained for the rat adrenal gland by this method were highly unreliable. The problem was the small size fo the rat adrenal. In a mature intact animal of 200 g body weight, the adrenal gland weighs ~ 20 mg. Fourteen days after $\bar{H}$ the gland can weigh less than 2 mg. It was found that evaporation of the buffer solution during the weighing of the gland could be greater than 1 mg in less than 1 minute. Consequently, the method was not practical for volume determinations of very small objects. Weighing the adrenal in a closed vessel of known volume containing a liquid of known specific gravity (pycnometry) would be a much better method. This method also has the advantage that the density of the gland can also be determined.

VI. Statistical Treatment of Data

The data from 5 blocks for each animal were totaled except for the animals hypophysectomized 10 and 14 days earlier where the data from 3 blocks were totaled. The values for either relative volume or surface density were obtained utilizing the appropriate formulas for these parameters. From this information, the mean, the standard deviation from the mean and the standard error of the mean were determined for the 5 animals in each of the time periods. The means of different time periods were compared by applying the two sided T distribution test. Two means were considered significantly different if the probability of error, P, was less than 0.05.

RESULTS

I. The Fine Structure of the Zona Fasciculata of the Hypophysectomized Rat

It must be remembered that the principal distinguishing characteristic of the fasciculata of the intact rat is the appearance of the mitochondria. The mitochondria are usually spherical and possess vesicular cristae for the most part (Figures 1 and 2).

As early as one day after hypophysectomy, there is a dramatic increase in the size and number of lipid droplets appearing in the cytoplasm of fasciculata cells (Figure 7). This increase continues but is more gradual in the succeeding days up to 14 days after hypophysectomy. Two rather subtle changes are (1) the sudden appearance of dense granules in a few mitochondria and (2) an increased incidence of tubular cristae occurring in some of the mitochondria (Figure 8). There are no other obvious changes in the ultrastructure of zona fasciculata cells taken from animals hypophysectomized 24 hours earlier.

Two days after hypophysectomy, there is still little obvious change when the cells are compared to fasciculata cells from intact rats. Figure 9 is a low magnification micrograph illustrating this point. An interesting finding is the presence of what appear to be two unmyelinated nerve fibers (arrows), probably of the autonomic nervous system. Nerve fibers are rarely found in the adrenal cortex and their function is unknown. Figure 10 demonstrates the increasing frequency of mitochondria bearing vesiculo-tubular or tubular cristae (upper right corner). Figure 11 is a high magnification view of fasciculata cytoplasm two days after hypophysectomy showing the tubular shape of the SER, the presence of two small residual bodies, and the presence of some intramitochondrial tubules (arrow). The later structures are not uncommon in fasciculata mitochondria of intact or hypophysectomized animals.

After the animal has been deprived of ACTH for four days, the mitochondria exhibit great variation in shape and size, and tubular cristae are becoming more evident (Figure 12). As a matter of fact, tubular cristae are most prevalent in mitochondria of fasciculata cells of animals hypophysectomized 4 or 10 days previously, while mitochondria of fasciculata cells in animals hypophysectomized 14 days earlier possess cristae that are, again, more vesicular than tubular. Figure 13 is a high magnification micrograph of the fasciculata cytoplasm showing the relative abundance of SER as well as a bit of the scarce RER (arrow). The cell membrane and dense granules within the mitochondria are easily visualized.

At ten days following hypophysectomy, most nuclei of fasciculata cells are no longer spherical as the cells continue to atrophy. One of these irregular nuclei is depicted in Figure 14. In another micrograph (Figure 15) can be seen the great number of lipid droplets that have accumulated in many of the cells at this time. The variation in mitochondrial size and morphology is also apparent here. Both large and very small spherical mitochondria containing vesicular cristae can be seen. Ovoid and other irregular mitochondria possessing vesicular and tubular cristae are also encountered. Examples of this mitochondrial morphology are seen in the lower left corner of Figure 15. Figure 16 is a high magnification

XUM

micrograph of some irregularly shaped mitochondria with vesiculo-tubular and tubular cristae which are frequently encountered at this time. Notice the dense granules within the mitochondria and the still quite numerous profiles of SER. Another micrograph (Figure 17) illustrates the small variably shaped mitochondria also found in fasciculata cells of rats hypophysectomized for 10 days. These mitochondria possess mostly vesicular cristae. Also shown here are lipid droplets and several residual bodies. Rarely, a giant mitochondrion is found, but in the overall view, there is a general reduction in mitochondrial size beginning ten days after hypophysectomy. Dense granules which have been frequently mentioned, seem to reach their peak in numbers at this time and then decline quite sharply at 14 days after hypophysectomy. A curious finding was the rather unusual arrangement the SER occasionally assumes. A considerable width of parallel arrays of the membranes of the SER seemingly flow in river-like fashion through the cytoplasm to the exclusion of most other organelles. One of these "rivers" of membrane can be seen in Figure 18. This condition is by no means confined to this time period for it is also observed in the fasciculata cells of intact 1, 4 and 14 day hypophysectomized rats. This is a rare finding at all these time periods but the arrays of SER membrane appear to be wider in the cells of rats hypophysectomized 10 or 14 days earlier.

An interesting phenomenon occurs at this time -- the fasciculata and reticularis blend together into one zone so that the distinction between these two zones which was based on mitochondrial morphology no longer exists. Residual bodies become quite large and more of these are present in the cells close to the medulla than in the more superficial layers of the zonae fasciculata-reticularis.

Fourteen days after pituitary removal, several established trends continue. Lipid accumulates as before so that most cells are almost completely filled with this substance and mitochondria become even smaller in size. These two characteristics are well illustrated in the low magnification micrograph of Figure 19. Note the presence of vesicular cristae in the mitochondria, a consistent finding at this time. Figure 20 demonstrates the amount of cellular atrophy that has occurred fourteen days after hypophysectomy. Almost the entire field of this low magnification micrograph is occupied by one fasciculata parenchymal cell. A similar parenchymal cell from an intact animal would occupy at least twice this area. Note the small size and the variable shape of the mitochondria and that their cristae are vesicular or vesiculo-tubular in configuration. Surprisingly enough a considerable abundance of SER is still present. The nucleus of this cell is less shrunken than is usually the case. This illustrates the general point that there are differences between cells and the rate at which they exhibit atrophic changes. These differences are averaged out in the morphometric analysis. Figures 21 and 22 demonstrate some of this variation. In Figure 21 note that the mitochondria are extremely small but still possessing mostly vesicular cristae. The SER is quite poorly developed. Figure 22 is a micrograph of another cell at the same magnification as Figure 21 and from the same time period. It also contains small mitochondria with cristae of variable type but note the enormous quantity of SER membranes arranged in parallel arrays "streaming" through the cytoplasm. For a cell to harbor this huge quantity of SER after being deprived of ACTH for so long a period is indeed a remarkable finding. It must be repeated, however, that this condition is of rare occurrence.

Certain other changes were noted at various times after hypophysectomy, but no clear patterns emerged from these observations. Degenerating mitochondria were occasionally seen inside lysosomal structures beginning the first day after hypophysectomy. A peak in their frequency seemed to be reached at 2-4 days with very few seen at the 10 and 14 day periods. Cholesterol crystals were sometimes observed in the cytoplasm of fasciculata parenchymal cells. These structures reached their maximum number 2-4 days postoperatively and sharply declined thereafter.

II. Morphometry

A. Relative Volumes

The relative volumes of the nuclei, cytoplasm and several subcellular organelles in fasciculata parenchymal cells are given in Table 1. The standard error of the mean (S.E.M.), percent difference* (Δ %), and probability of error (P) were calculated by standard statistical methods. The values for the means of the various time periods following hypophysectomy were compared with those of the intact controls and P was considered significant if its value was less than 0.05. A graph of these same data is shown in Figure 23.

---

*Percent difference means the difference between a number and a base as calculated by the formula $\frac{\text{number-base}}{\text{base}}$.

It can be seen that the relative volume of the mitochondria increases slightly one day after hypophysectomy and then steadily declines with time, although there is no statistically significant difference in the values until 10 days after hypophysectomy. The lipid relative volume demonstrates a highly significant increase the first day after hypophysectomy. It continues to rise at a rapid rate until the fourth day when the rate of increase slows but the relative volume continues to increase until it reaches a maximum on the fourteenth day after hypophysectomy. This represents an increase of about ten fold over the control value with more than half of the total increase occurring in the first day. The relative volume of the endoplasmic reticulum declines at first but then rises slightly. At 14 days after hypophysectomy there is no significant difference from the control value. The relative volume of the cytoplasmic matrix exhibits a general decline although it does fluctuate somewhat. The relative volumes of nucleus and cytoplasm were not graphed but the table shows that the nuclear relative volume increases to a maximum at 2-4 days after hypophysectomy and then declines thereafter. The 2 and 4 day periods exhibit highly significant increases ($P < 0.005$) and the 14 day time period represents a significant decrease ($P < 0.05$) as compared to the control value. Since the relative volumes of nucleus and cytoplasm add up to 100%, the relative volume of the cytoplasm varies inversely with the relative volume of the nucleus.

XUM

B. Plasma Levels of Corticosterone

The results of the plasma corticosterone determinations are given in Table 2. These values are expressed as μg per 100 ml of plasma. By the first day after hypophysectomy, there is a 70% decrease in circulating corticosterone levels. Another 10% decrease occurs on the following day after which time a gradual increase is observed until the fourteenth day. However, this increase from the second to the fourteenth day is not statistically significant ($P > 0.1$). It can be said, therefore, that there is no change in circulating corticosterone levels after the second day following hypophysectomy.

C. Absolute Volumes

The absolute volumes for the morphometric parameters of fasciculata parenchymal cells are given in Table 3. These values are expressed as μm^3 with respect to the cytoplasm of an average fasciculata parenchymal cell except for surface density which is expressed as μm^2 in relation to the cytoplasm of an average fasciculata parenchymal cell. As with relative cell volume, the standard errors (S.E.M) have been computed as has the percent difference (Δ%) and the P values. The last two quantities were calculated in relation to the control values.

Absolute cell volume has been graphed and is shown in Figure 24. There is a highly significant decrease of 32% in the first day after hypophysectomy. The sharp decline continues, reaching a minimum volume at four days. A slight, gradual rise occurs from the fourth to the fourteenth day. There is a total decrease in absolute cell volume of 62% when the fourteenth day following removal of the pituitary is compared to the control value. The absolute nuclear and cytoplasmic volumes were not graphed but their decline closely parallels to that of the absolute cell volume as would be expected since the latter value is the sum of the nuclear and cytoplasmic volumes. The absolute volumes of mitochondria, lipid, endoplasmic reticulum (ER) and cytoplasmic matrix have been graphed along with the corticosterone plasma levels for purposes of comparison in Figures 25 and 26. It is evident that the curves for mitochondria, ER and cytoplasmic matrix all decline in rather close agreement with the curve for corticosterone plasma levels. Generally speaking, all of these components sustain highly significant decreases in the first postoperative day and tend to reach a minimum on or about the fourth day after hypophysectomy. The absolute lipid volume undergoes a highly significant increase of 342% in the first day and then generally declines although it is still more than 300% above the control value on the fourteenth day after hypophysectomy. Comparing the means for the fourth and fourteenth day of the lipid curve reveals no significant difference ($P > 0.05$). There is a significant 30% decrease in the absolute volume of the mitochondria in the first day following hypophysectomy. This represents about 40% of the total decrement in mitochondrial volume as most of this decrease occurs by the fourth day. The absolute volume of the ER also declines rapidly with a 36% decrease occurring in the first day. This is a highly significant decrease ($P < 0.005$) and virtually all of the decrease occurs by the second day after hypophysectomy. In a similar fashion, the cytoplasmic matrix shows a highly significant decrease of more than 50% in the first day and reaches its minimum volume by the fourth day. However, the slow increase in volume seen from the fourth to the fourteenth day is of statistical significance ($P < 0.005$).

Figure 27 is a graph which compares the decline in corticosterone plasma levels and the surface density of the ER (expressed in μm^2) with time after hypophysectomy. It is evident that a highly significant decrease of about 50% occurs in the first day and, as with most of the other absolute parameters, a minimal value is reached by the fourth day. The agreement between the corticosterone and ER curves is quite striking. Thus, the surface area occupied by the endoplasmic reticulum appears to be the best predictor of secretory activity in these cells.

III. The Fine Structure of the Zona Intermedia of the Hypophysectomized Rat.

In their light microscopic investigation of the zona intermedia, Cater and Lever (1954) mentioned that this zone is poorly defined or completely absent between the 42 and 182 days of life in rats of both sexes. I found this to be true for the intact 51-52 day old male rats used in the present study. Since this zone is absent in these rats, no electron micrographs of this zone were obtained. However, beginning on the fourth day following pituitary removal, the zona intermedia becomes apparent and by the fourteenth post-operative day it is a wide band of lipid-free cells.

In general, the fine structure of the cells of the intermediate zone of the hypophysectomized animal resemble that of the cells of both the glomerulosa or fasciculata of the intact animal with the following exceptions: (1) reduced number of lipid droplets, (2) possible reduction in the number of Golgi complexes and (3) variation in the mitochondria. Although no quantitative studies were conducted to verify the second point, it was my impression that there appeared to be fewer Golgi complexes present than in either glomerulosal or fasciculata cells of intact animals.

The third point warrants further explanation. Most mitochondria in the zona intermedia are smaller and more variable in their shape and also more variable as to type of cristae than those mitochondria from the zonae glomerulosa and fasciculata of an intact animal (compare Figure 1 with Figure 27, for example). Many mitochondria are spherical, some are elongated and still others are irregular in outline. Many cristae are tubular, while others are vesicular to vesiculo-tubular. Although mitochondrial shape can vary within the same cell, the type of cristae within the mitochondria usually remains the same in a given cell. The appearance of dense granules, usually assumed to be deposits of polyvalent cations (Christensen and Gillim, 1969), within the mitochondria seems to be a rather constant feature of zona intermedia cells, particularly in the later days after hypophysectomy.

Four days after hypophysectomy, when the intermediate zone is only 2-4 cells wide, those cells bordering on the zona glomerulosa tend to resemble glomerulosa cells, that is, the mitochondria are oval to elongated in shape and possess mostly tubular cristae (Figures 28 and 29). Those cells of the intermediate zone that border on the zona fasciculata tend to contain mitochondria that are spherical or irregular in shape and possess vesicular cristae (Figures 30 and 31).

Ten days after hypophysectomy, the situation described above becomes even more pronounced. At this time, the intermediate zone is a wide band which is many cells thick. Those cells of the intermedia in the vicinity of the zona glomerulosa possess irregular mitochondria containing mostly tubular cristae. This can be clearly seen in the micrographs of Figures 32 and 33. Those intermedia cells adjacent to the zona fasciculata exhibit characteristics of fasciculata cells from intact animals, i.e. spherical mitochondria possessing mostly vesicular cristae (Figures 34 and 35). In the middle of the zona intermedia, both types of cells exist side by side. This is especially well illustrated in Figures 36 and 37. The cell in the left side of either micrograph resembles a glomerulosa cell from an intact animal while the cell to the right resembles a fasciculata cell from an intact animal. Notice also the absence of lipid droplets which is the main distinguishing feature of the zona intermedia.

This is not to say that the above description invariably prevails, for cells can be found containing mitochondria bearing both tubular and vesicular cristae at all levels of the zona intermedia. However, the general trend is for cells of the intermedia adjacent to the glomerulosa to resemble cells of the glomerulosa of intact animals and for intermedia cells in the vicinity of the fasciculata to resemble cells of the fasciculata from intact animals. Thus, the terms intermediate or transitional zone are particularly appropriate, for the cells truly appear to be intermediate in structure between those of the two outer cortical zones.

What has just been said for the 10 day period can be repeated for the period 14 days after hypophysectomy. The only difference observed is that the zone is slightly wider at this time than at 10 days. Figure 38 is a low magnification micrograph taken from the middle of the zona intermedia of a rat hypophysectomized 14 days previously. It well illustrates the diversity in

XUM

in mitochondrial morphology displayed by this zone. There are spherical, ovoid, elongated and even a branched mitochondrion in this field (arrow). Cristae run the gamut from vesicular, vesiculo-tubular to tubular in form. Figure 39 which is a higher magnification view from the same area serves to demonstrate the moderate amount of SER present (arrow). Other arrows point out an accumulation of glycogen particles and also dense granules present within mitochondria.

XUM

DISCUSSION

I. The Zona Fasciculata of the Rat After Hypophysectomy

This work has shown, by quantitative morphometric methods, that the quantity of lipid in zona fasciculata cells rises after hypophysectomy. In addition, the absolute volume of mitochondria and the endoplasmic reticulum as well as the surface area of the endoplasmic reticulum declines in these cells after pituitary removal. These data confirm the findings of numerous authors (Deane, 1962; Nishikawa et al., 1963; Fujita, 1972; Canick and Purvis, 1972). However, these opinions have not been supported by quantitative data, and therefore, this study is an extension of their work.

The sudden rise in the number of lipid droplets within the cells that occurs as soon as one day after hypophysectomy is a direct reflection of the physiologic state of the zona fasciculata. It has been demonstrated that the principal constituents of the lipid droplets are cholesterol and cholesterol esters (Deane, 1958; Moses et al., 1969). Since cholesterol is the precursor from which all of the adrenocortical hormones are synthesized (Christensen and Gillim, 1969) and ACTH is necessary for steroidogenesis (Farese, 1968) the absence of ACTH results in the cessation of hormone synthesis and the consequent accumulation of cholesterol in the form of lipid droplets within the cytoplasm. ACTH also stimulates the synthesis of cholesterol by adrenocortical cells (McKerns, 1968); thus, hypophysectomy would prevent this contribution to the lipid droplet pool, but, in the case of the rat, this contribution is of a minor nature (Morris and Chaikoff, 1959; Ichii et al., 1967). Certainly, in the present study, it appears that the

XUM

cessation of corticosteroidogenesis far outweighs the effects of the cessation of cholesterol synthesis by fasciculata cells as evidenced by the rapid accumulation of lipid droplets within these cells following pituitary removal. Fujita (1972) not only believes that lipid accumulation in adrenocortical cells of hypophysectomized rats is due to cessation of hormone synthesis but also to inhibition of hormone release from the cell as well. Fujita further suggests that adrenocortical atrophy is primarily caused by fatty degeneration of cells of the fasciculata and reticularis. Fatty degeneration is a pathological term meaning simply degeneration of cells due to an accumulation of fatty deposits within them. In the present investigation, it was observed that there was more atrophy of fasciculata cells of hypophysectomized rats that were heavily infiltrated with lipid droplets than in those cells containing fewer lipid droplets. However, severe cell atrophy was observed in most fasciculata cells 10-14 days after hypophysectomy regardless of their lipid content so that I believe that the primary cause of adrenocortical atrophy after hypophysectomy is the lack of trophic stimulation by ACTH and secondarily a possible fatty degeneration of these cells.

Much of the work on the ultrastructure of the adrenocortex of the hypophysectomized rat done prior to 1966 is subject to criticism and doubt due to the poor fixation and embedding techniques employed. In the present investigation, for instance, I saw no instances of cristae protruding from mitochondria or of "open" mitochondria as previously reported (Sabatini et al., 1962; Borowicz, 1965). These phenomena can probably be considered as artifacts of improper fixation. On the other hand, the work of Fujita (1972) demonstrates good preservation of cytological detail, and some of his observations are directly comparable to those of the present investigation. His observations that there was a great accumulation of lipid droplets and that mitochondrial shape and size became quite irregular after hypophysectomy agree quite closely with my findings. He also described the nuclei of fasciculata parenchymal cells as decreasing in size and becoming irregular in shape 10 days after hypophysectomy and my observations 10-14 days after hypophysectomy are in agreement with this finding. However, he found that almost all cristae were converted from the vesicular to the tubular shape 10-25 days after hypophysectomy whereas I found the incidence of tubular cristae to be at a maximum 4-10 days after hypophysectomy with a decline thereafter. At 14 days after hypophysectomy, I observed most cristae to be vesicular once again. Fujita reported a considerable reduction in the amount of SER per cell 10-25 days after hypophysectomy as compared to the cells of intact controls. The absolute volume of the ER also declines in my study, but morphometric analysis of the relative volume of the ER showed a significant increase over the control value at 10 days after hypophysectomy but then a decline occurred at 14 days to a value not significantly different from the intact control value. Some of the discrepancy between the two results may be attributed to the fact that Fujita's description is an impression gained from studying selected fields while the present results were obtained by the morphometric analysis of hundreds of random micrographs so that variations in the quantity of ER from cell to cell were averaged out. Then too, Fujita's description is limited to the quantity of SER whereas my category of ER includes SER, RER and the Golgi membranes

II. Morphometry

Many of the previous morphometric studies conducted on the rat adrenal cortex after various experimental procedures report only relative volumes of cellular components (Magalhães and Magalhães, 1969; 1972) or absolute volumes expressed in relation to quantities other than cell or cytoplasmic volume (Yago et al., 1971; Sekiyama et al., 1971). It is, therefore, difficult to make complete comparisons of all of these data with the data from the present investigation which is expressed in both relative and absolute volumes in relation to the cytoplasm of a fasciculata parenchymal cell. Since relative volumes can be calculated from absolute volume data, it is deemed advisable to discuss only absolute volumes in any great detail here. The studies of Nussdorfer and co-workers (e.g., Nussdorfer and Mazzocchi, 1970) do contain absolute volumes but, as was previously discussed, their papers never contain data for the cytoplasmic matrix compartment. For this reason, their data cannot be directly compared to mine and thus will not be discussed extensively. On the other hand, Mäusle and Scherrer (1974) conducted a morphometric study on the effect of sex hormones on the adrenal cortex of the rat in which are given absolute volumes relating to the zona fasciculata cells of intact male rats which are directly comparable to my data. However, no weight or age of the rats is given. Mäusle and Scherrer report an average cell volume that is much smaller than mine (1607.7 vs. 2465.8 μm^3) and a correspondingly smaller mitochondrial volume (551.9 vs. 943.9 μm^3). Perhaps much younger animals were used in their study. However, the absolute volumes of the ER agree rather closely (222.1 vs 275.4 μm^3). The greatest difference between the two studies lies in the area of absolute lipid volumes where they found about four times as much lipid as I did (194.7 vs. 48.9 μm^3). It is possible that my animals were more stressed either chronically, because of excessively noisy animal towers, or acutely at the time of killing. Stress will deplete lipid stores because increased amounts of glucocorticoids are produced (Deane, 1962). The surface density of the ER was not analyzed in the study of Mäusle and Scherrer (1974). In reviewing the differences in the morphometric data, I would have to agree with the comments of Mäusle and Scherrer (1974) that the values obtained for a morphometric investigation are probably only comparable to those values obtained under completely identical experimental conditions in the same laboratory.

The differing rates of atrophy of the several cellular components can be demonstrated by the percent change in absolute volumes (Table 3). For example, at day one the mitochondrial volume shows less change than the cytoplasmic volume does, then it shows more change on the following days. Hence, the relative mitochondrial volume at first increases then decreases which means that the mitochondrial volume declines more rapidly than does cellular volume at day two and thereafter. The absolute volume of the ER is seen to shrink faster than the absolute cytoplasmic volume at first and then more slowly on succeeding days. The surface density of the ER shrinks the fastest of all the cellular values and, therefore, this rapid decline in surface area may become rate-limiting for steroidogenesis.

Cell fractionation studies have been the principal source of information about the localization of biosynthetic enzymes in steroid-secreting cells of the adrenal cortex. These studies have shown that the enzymes responsible for cholesterol synthesis within the adrenocortical cell reside in the microsomes or nearby cytoplasm (Christensen and Gillim, 1969). ACTH does stimulate cholesterol synthesis from acetate in these cells (McKerns, 1968) but, at least in the rat, this source of cholesterol for steroidogenesis does not seem to play a major role. Morris and Chaikoff (1959) found that in the intact rat 92-98% of the total adrenal cholesterol is taken up from the plasma. Also using the rat, Ichii et al. (1967) found a slightly smaller amount of adrenal cholesterol coming from the plasma (80-90%) with the remainder being derived from in situ synthesis.

Cell fractionation studies have also demonstrated that the enzymes responsible for corticosteroidogenesis are located in both the mitochondrial and microsomal fractions (Samuels and Uchikawa, 1967). These studies have further demonstrated that most of these enzymes are found in the microsomal fraction (Samuels and Uchikawa, 1967) which is probably a mixture of the SER, RER, and Golgi elements. The microsomes, then, probably correspond to my category of the ER. Figure 26 shows that the absolute volume of the ER declines steadily until the second day after hypophysectomy with virtually no change therafter. This general pattern of decline in ER volume after hypophysectomy was also seen by other morphometric investigators (Nussdorfer and Mazzocchi, 1972; 1973) but detailed comparisons cannot be made because of differences in the methods of presenting the data.

Figure 25 illustrates how the absolute volume of the mitochondria declines to the fourth day after hypophysectomy and then undergoes no further change. The Δ% figures of Table 3 show, however, that the absolute mitochondrial volume has

XUM

not declined as rapidly as the absolute cytoplasmic volume at day one indicating an increase in mitochondrial relative volume at that time period although the relative volume then declines steadily thereafter.

As was mentioned before, cell fractionation studies have shown that while most of the enzymes for corticosteroidogenesis occur in the microsomal fraction, some occur in the mitochondrial fraction (Samuels and Uchikawa, 1967). Hirshfield and Koritz (1964) reported that mitochondrial swelling is an indication of increased pregnenolone synthesis in adrenal mitochondria. Since pregnenolone is the first step in steroid formation from cholesterol (Samuels and Uchikawa, 1967), mitochondrial swelling may indicate increased availability of this precursor. Koritz (1968) states that mitochondrial swelling itself is probably only the manifestation of an underlying modification of mitochondrial membranes which may be the critical factor in the stimulation of pregnenolone synthesis.

Even though a slight increase in mitochondrial volume was detected, which may reflect mitochondrial swelling, it is nonetheless true that the absolute volume of this organelle declines. Thus, the absolute amount of enzyme available for hormone biosynthesis probably also declines and results in decreased corticosterone production.

Other instances of mitochondrial swelling after hypophysectomy have been reported in the literature. Sekiyama et al. (1971) reported an increase in the average volume of an individual mitochondrion but a decrease in the average number of mitochondria per 100 μm^3 of parenchymal cell cytoplasm of male rates three days after hypophysectomy. They also reported an increase in the relative volume of fasciculata mitochondria two and three days following hypophysectomy.

The authors speculated that this swelling of the mitochondria after hypophysectomy must be due to an entirely different process than that which causes mitochondrial swelling occurring after administration of ACTH as reported in their companion paper (Yago et al., 1971). Apparently, they felt that it was paradoxical that an increase in ACTH (via injection) and a decrease in ACTH (via hypophysectomy) should both produce mitochondrial swelling. They theorized that a certain small amount of ACTH is necessary to maintain the structural equilibrium of fasciculata mitochondria and hypophysectomy produces a disruption of this equilibrium.

Canick and Purvis (1972) in their study of mitochondrial size in the fasciculata cells of the intact and hypophysectomized female rat used the same method as used by Sekiyama et al. (1971) for quantitatively analyzing changes in the mitochondrial population. They also found an increase in mitochondrial size and a decrease in mitochondrial number in relation to a given area of fasciculata cytoplasm 4, 7, 11, and 14 days after hypophysectomy. Their data are not presented in relation to cytoplasmic or cellular volume so that direct comparisons with the data of the present study are not possible.

The morphometric data of Nussdorfer and Mazzocchi (1972, 1973) that pertain to the hypophysectomized rat cannot be directly compared to the data of the present investigation for reasons previously stated, but the general trend reported for the absolute mitochondrial volume was a decline after hypophysectomy.

Most investigators report an increase in the lipid content of the fasciculata following hypophysectomy (Deane, 1962; Nishikawa et al., 1963; Fujita, 1972; Canick and Purvis, 1972). The present quantitative investigation confirms this result (Table 3 and Figure 25). Since corticosterone production has virtually

XUM

ceased following hypophysectomy,* lipid droplets, composed mostly of cholesterol and cholesterol esters which are the precursors for the synthesis of corticosterone (Deane, 1958; Moses et al., 1969), accumulate in the fasciculata and reticularis. The cause of this accumulation is presumed to be a loss of the membranes of the mitochondrial cristae and the SER and a loss or inactivation of the associated enzymes, which are responsible for corticosterone synthesis (Christensen and Gillim, 1969). ACTH does stimulate cholesterol synthesis but as was pointed out before, at least for the rat, this source of cholesterol appears to play a minor role in steroidogenesis. As the quantity of mitochondria and SER decreases after

*Tchen (1968) points out that the fluorescent technique for measuring plasma levels of corticosterone (as was used in the present study) is not strictly specific for corticosterone and the basal level of secretion seen after hypophysectomy is due mostly to secretion from the zona glomerulosa (aldosterone). More sophisticated techniques such as a competitive protein binding technique as used by Colby et al. (1974) have shown, for example, that the plasma levels of corticosterone can decline from 14.6 μg/100 ml in the intact female rat to 0.8 μg/100 ml one week after hypophysectomy. A drop from 14.6 μg/100 ml to 2.3 μg/100 ml is seen the first day after hypophysectomy.

hypophysectomy, cholesterol utilization declines as well allowing unused cholesterol to accumulate in the cells in the form of lipid droplets. Mostafapour and Tchen (1971) have shown that the capacity of the rat adrenal cortex to produce corticosterone in vitro declines to a very low level within 6-12 hours after hypophysectomy. Quantitative morphometric studies of adrenocortical cells have not been performed for these early time periods but the results of such a study should be informative. Nussdorfer and Mazzocchi (1972, 1973) are among the few investigators who find a decrease in the lipid content of the zona fasciculata in the rat after hypophysectomy. They also report finding virtually no qualitative changes in fasciculata cells of rats 9-10 days after hypophysectomy as compared to the intact control rats. This lack of qualitative ultrastructural alteration 9-10 days after hypophysectomy is contrary to the findings of the present investigation as well as to those of Fujita (1972) and Canick and Purvis (1972). It would seem that the completeness of hypophysectomy is doubtful in the work of Nussdorfer and Mazzocchi although they state in their 1973 paper, but not in their 1972 paper, that this point was checked by the interruption of the animal's growth, by the decrease in adrenal size, and by direct examination of the sella turcica with a dissecting microscope. No corticosterone plasma level determinations were done in these two studies. Be that as it may, their data cannot be compared with the data of the present investigation for the reasons stated previously.

I believe that caution should be exercised with respect to two problems dealing with morphometric analysis of steroid-secreting cells. First of all, one should be cautious about the interpretation of morphometric data when only relative values are given. It has already been pointed out how the relationship between the rate of cell shrinkage and the rate at which a cellular component is changing can turn a real increase into an apparent decrease and vice versa. Because of this, I think that both relative and absolute values are necessary in order to accurately evaluate what is occurring within cells subjected to experimental manipulation. Of course, relative values can be calculated from absolute values, thus the presentation of only absolute values is an acceptable procedure. Secondly, I feel care should be exercised in choosing a single morphological parameter as the most important indicator of the functional activity of a steroid-secreting cell. The selection of the quantity of SER for this indicator as advocated by Nussdorfer (1970a) seems debatable at least in regards to the present investigation. For one thing, present data indicate a general decline in both mitochondrial relative and absolute volumes (Figures 23 and 25). It can be seen that the absolute mitochondrial volume curve follows the corticosterone curve more closely than the absolute ER volume curve does after hypophysectomy (Figure 25). For another, if a single morphological parameter had to be chosen as an indicator of the functional activity of a fasciculata parenchymal cell for the present study, it would have to be the surface density of the ER (Figure 27). Here is found the closest agreement between the curves for any of the absolute values and the curve for the corticosterone plasma determinations. However, I think that it is unwise to select any one single parameter to serve that function. Since the mitochondria, ER, and lipid droplets are all implicated in steroidogenesis, I think that the way that the quantities of all these components interact within an adrenocortical cell is the proper indicator of the physiological activity of that cell

It is evident that further studies are needed on the ultrastructural alterations induced by hypophysectomy on rat adrenocortical cells. For instance, it would be interesting to do a morphometric study on the Golgi apparatus to see if the quantity of this organelle actually diminishes after hypophysectomy as has been assumed. The results might shed some light on the function of the Golgi apparatus in a steroid-secreting cell which, at the present time, is obscure. Morphometric studies should be conducted on residual bodies to better define their role in degenerating adrenocortical cells.

III. The Zona Intermedia

The zona intermedia only occurs as a well defined zone in the rat, cat, dog, sheep, rabbit and horse (Cater and Lever, 1954). Even in the rat, it is not present for the full life of the animal as it cannot be clearly identified in the adrenals histologically or histochemically during the 6th to the 26th week of life although it is present before and after this time (Cater and Lever, 1954). These authors also suggest that the presence or absence of the intermediate zone depends on the functional activity of the anterior pituitary, for when a hypophysectomized rat is treated with ACTH, the lipid-free cells of the zona intermedia soon fill with lipid making them indistinguishable from the lipid-containing cells of the zona fasciculata. Once again, it must be remembered that the chief distinguishing characteristic of the zona intermedia is that it contains a very small quantity of lipid droplets. For the above reasons, some authors do not mention the zona intermedia in their studies of the adrenocortex of the rat while others have included it with either the glomerulosa or fasciculata of the rat's adrenal cortex.

Controversy surrounds the origin and the function of the intermediate zone. Mitchell (1948) using colchicine to arrest mitosis in metaphase, saw very few mitotic figures within the zona intermedia and considered this zone to be an area of nondividing cells. Many authors agree that the center or centers of cell proliferation in the adrenal cortex reside in either the glomerulosa or outer fasciculata (Hoerr, 1931; Mitchell, 1948; Cater and Lever, 1954; Walker and Rennels, 1961; Wright, 1971). Mitchell theorized that cells proliferating from the outer fasciculata forced cells against other rapidly dividing cells from the inner glomerulosa creating a band of small compressed cells, his so-called "zone of compression", which corresponds to the zona intermedia. Cater and Stack-Dunne (1953) also described the intermedia as a mitotically inert zone. Cater and Lever (1954) counted mitotic figures in the rat's entire adrenal cortex and found the fewest number of cells in mitosis in the zona intermedia. Their investigation revealed the greatest number of mitotic figures to be in the outer fasciculata and in the inner glomerulosa. This evidence prompted them to suggest that the zona intermedia may be formed as the result of rapid cell division in these two areas. Ford and Young (1963) using tritiated thymidine, which is rapidly incorporated into replicating DNA, and autoradiography for detection of the presence of label, found much label and one fourth of all the mitotic figures in the intermediate zone of 6-10 day old rats. Most of the rest of the mitoses were found in the outer fasciculata and glomerulosa so that they considered these three regions together to be the center of cell generation in these

XUM

young rats. Wright (1971) also used tritiated thymidine to label the adrenocortical nuclei of 14 day-old male rats. He found the highest index of labelling to be in the "proliferative zone" which corresponds to the zona glomerulosa, the zona intermedia and the outer fasciculata. Reiter and Pizzarello (1966), again using tritiated thymidine, demonstrated the presence of numerous mitoses in the intermedia of four month-old male rats.

It can be concluded that the site of origin of cells replacing effete cells in the adrenal cortex has not been conclusively demonstrated. It is probable that this site is different in younger and older animals. The discrepancies with respect to the zona intermedia are most troublesome and should be resolved by further experimentation.

Cater and Lever (1954) proposed a possible function for the zona intermedia. They considered the zona intermedia to be a zone of capillary compression with the amount of compression being directly proportional to the degree of cell packing within the intermedia. The longer the time after hypophysectomy, the greater the cell packing, resulting in a decrease in the vascularity of the zona intermedia as compared to an intact rat. The authors believe that the anterior pituitary, by altering the degree of compression in the intermedia does, thereby, exercises some control over capillary blood flow. They further mention that fine nerves have been described in relation to the walls of the arteriae corticis in the outer layers of the adrenal cortex of the rat (Lever, 1952) and that it is probable that these nerves provide vasomotor control over the arteriae corticis. They suggest, therefore, that in the rat, rapid changes in blood flow through the

XUM

outer cortex could be mediated by vasomotor control of the arteriae corticis while a slower control of blood flow might be effected by the amount of capillary compression within the zona intermedia presumably under the influence of ACTH. In view of the fact that the zona intermedia increases in width with time after hypophysectomy, at least up to fourteen days, it would seem that a decreased blood flow in this zone as suggested by Cater and Lever would be incompatible with an increase in the width of this zone. On the contrary, decreased blood flow might lead to atrophy of the zona intermedia but it is evident that this is exactly what the intermedia does not do.

Although no quantitative studies have been done, the scarcity of lipid droplets, the decrease in number of Golgi complexes (present observations) and decrease in size of the mitochondria (present observation) in cells of the zona intermedia of the hypophysectomized animal as compared with cells of the zonae glomerulosa and fasciculata of the adrenocortex of the intact animal would seem to point to this zone as being one of cell inactivity.

On the bases of these observations, I would like to suggest a theory about the origin and function of the zona intermedia as well as to suggest some experiments to support or disprove this theory. I suggest that the zona intermedia is a dormant zone of inactive cells. Certain cells in the outer fasciculata and possibly the inner glomerulosa might possess an anti-atrophy factor such that when ACTH is at a low plasma concentration or absent from an animal these cells would not atrophy but would assume a dormant condition. I realize that there is no prior evidence for such a factor but, as yet, no one has looked for one. It is possible that the zona intermedia is not under control of ACTH but existing evidence indicates that it is. Cater and Lever (1954) specifically state that there is no doubt that the zona intermedia is subject to the influence of the anterior pituitary, for when hypophysectomized rats are treated with ACTH, the lipid-free cells of the intermedia become filled with lipid. Furthermore, if treatment is continued, the cells of the zona intermedia become indistinguishable from the lipid-filled zona fasciculata cells. This view that the zona intermedia is under control of ACTH is also shared by Dalton et al., 1944; Greep and Chester Jones, 1950; Vasama et al., 1957. In this dormant condition this band of lipid-free cells would be the zona intermedia. This anti-atrophy factor is repressed by ACTH so that when the plasma concentration of ACTH is increased or restored to the animal, the cells of the zona intermedia would be stimulated to differentiate into fasciculata or possibly into glomerulosa cells. The concentration of this factor would be greatest in cells in the outer part of the intermedia and decrease in an inwards direction, thus accounting for the alterations in width of this zone as plasma concentrations of ACTH varied. I am speaking of long term variations of ACTH, as for example, increases during periods of growth and decreases that accompany the aging process or an experimentally created deficiency as occurs after hypophysectomy as compared to short term variations that occur during periods of stress. Farese (1968) has shown that ACTH is necessary for the growth of the adrenocortex. Cater and Lever (1954) have demonstrated in the rat that the zona intermedia is present from birth to the 6th week of life, is absent between the 6th and 26th weeks of life and then reappears becoming increasingly prominent thereafter as the rat ages. Some insight into this problem might be gained if the plasma concentrations of ACTH were determined at the various time periods to to see if a correlation existed between the levels of ACTH and the presence or absence of the zona intermedia. According to this theory, the intermediate zone becomes increasingly more prominent as the rat ages probably because ACTH secretion is diminishing with age.

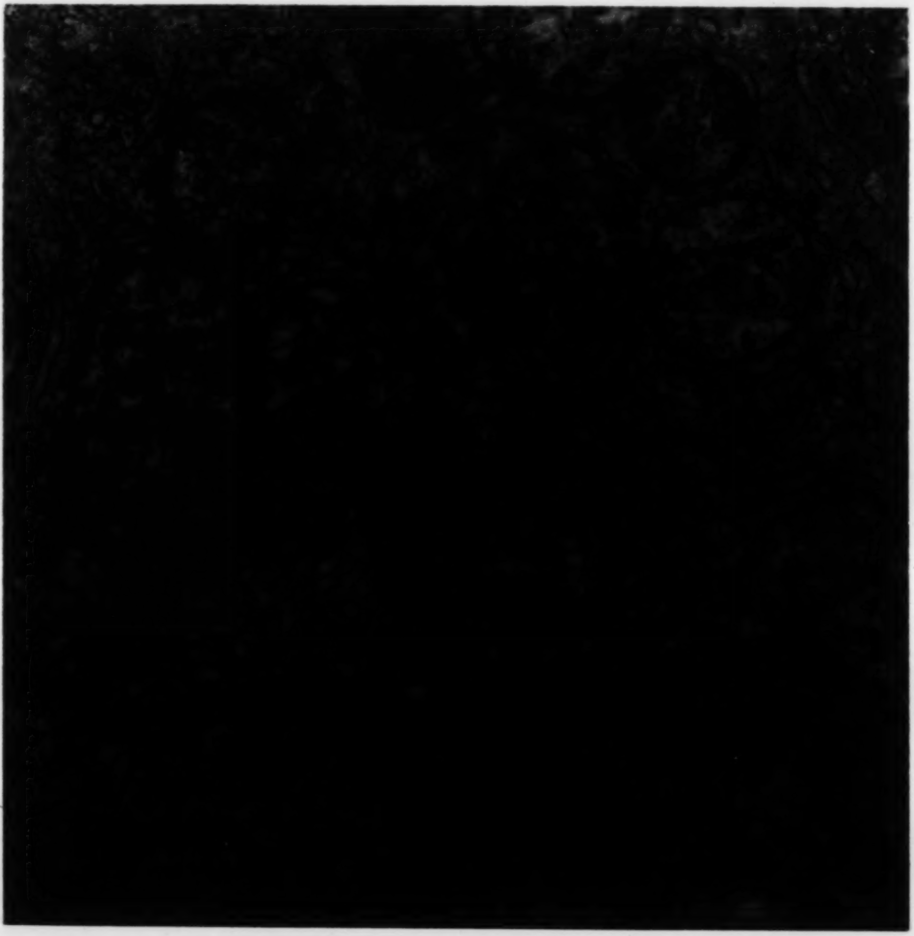
An experiment could be performed to see if the cells of the zona intermedia originate from existing cells or as a product of new cell division. Hypophysectomized rats injected with tritiated thymidine would be sacrificed 4, 10 and 14 days after surgery. Their adrenal tissues could then be studied by autoradiography to see if the adrenocortex contained label. If label were found in the cells of the zona intermedia, it would be evidence that these cells had arisen as a result of new cell division.

The presence of a zona intermedia would convey a certain survival value to an animal as it would supply a band of cells resistant to atrophy due to a decrease or removal of ACTH and would be a source of viable cells that could be quickly converted into fasciculata cells without immediate need of the slower cell division process.

Figure 1.	The zona fasciculata of the intact male rat. 14,000X

Figure 2. A higher magnification of the zona fasciculata of the intact male rat. 46,000 X

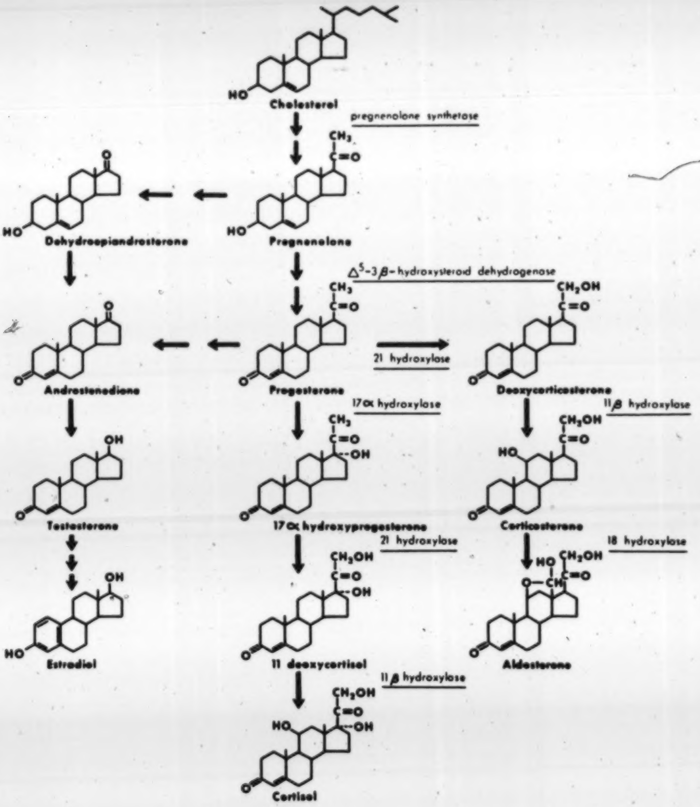
XUM



XUM

Figure 3. A diagram of the biosynthetic pathways of the principal corticosteroids.

XUM

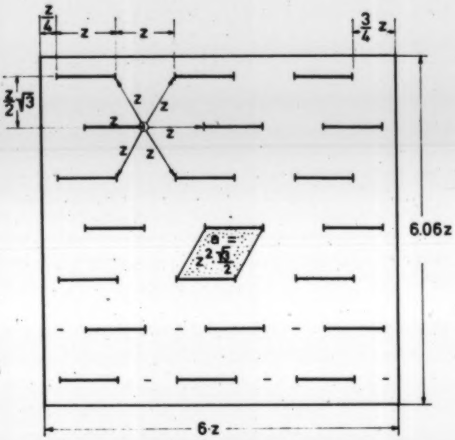


Enzyme	*Zonal Localization*	*Subcellular Localization*
Pregnenolone synthetase	All zones	Mitochondria
Δ^5-3β-hydroxy-steroid dehydrogenase	All zones	Microsomes
21 hydroxylase	All zones	Microsomes
11 β hydroxylase	All zones	Mitochondria
17 α hydroxylase	Fasciculata and reticularis	Microsomes
18 hydroxylase	Glomerulosa	Mitochondria

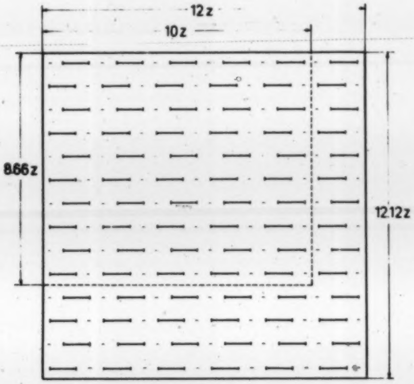
Diagram showing the pathways taken in the biosynthesis of the principal corticosteroids. The zonal distribution and subcellular distribution of the most important enzymes are given in the table.

Figure 4. An illustration of some of the test systems used in the technique of morphometric analysis.

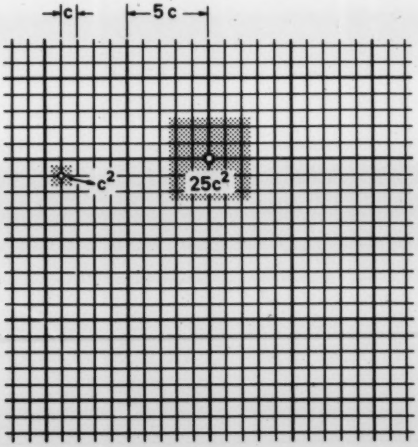
XUM


z/4
z
z
3/4 z
z/2 √3
z
z
z
z
z
z
a = z² √3/2
6.06z
6·z

A


12z
10z
8.66z
12.12z

B


c
5 c
c²
25c²

C

XUM

Figure 5. A graph illustrating the relationship between relative error in volume density estimation E_{V_V}, relative volume V_V, and test point number P_T.

XUM

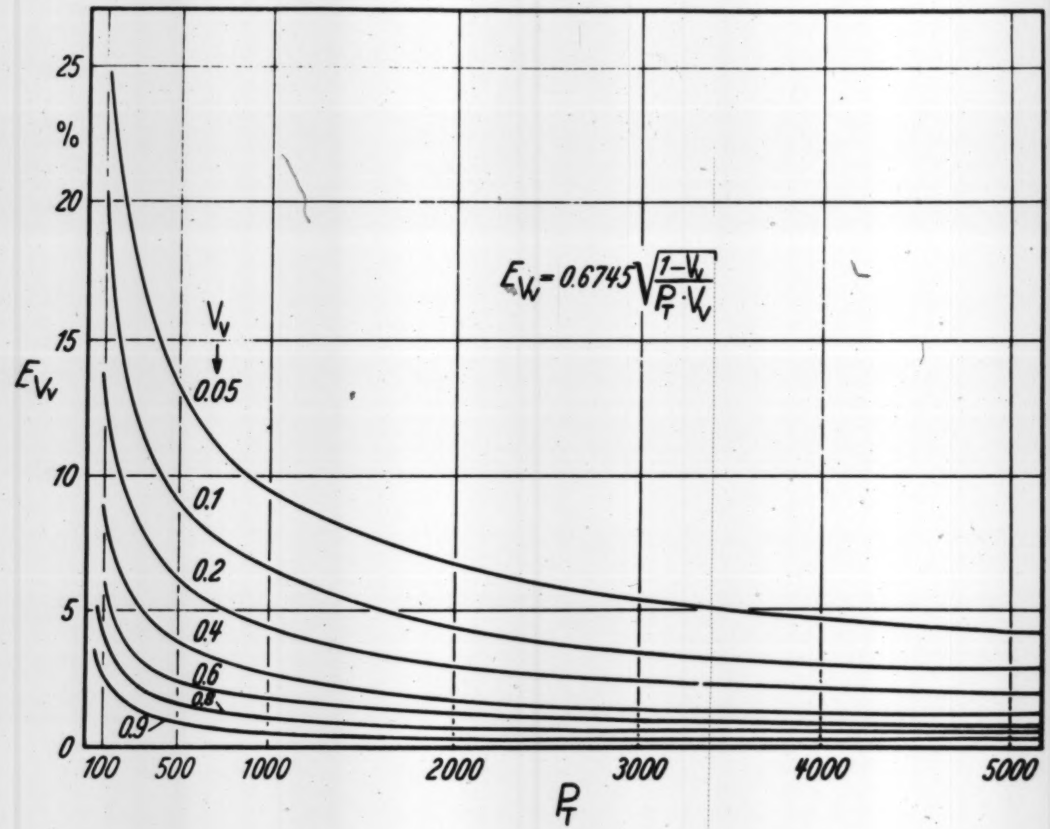

$E_{V_V} = 0.6745\sqrt{\frac{1-V_V}{P_T \cdot V_V}}$
V_V
0.05
0.1
0.2
0.4
0.6
0.8
0.9
E_{V_V}
%
25
20
15
10
5
0
100
500
1000
2000
3000
4000
5000
P_T

Figure 6. Morphometric model of the fasciculata parenchymal cell with morphometric parameters.

XUM

Morphometric Model of the Fasciculata Parenchymal Cell with Morphometric Parameters

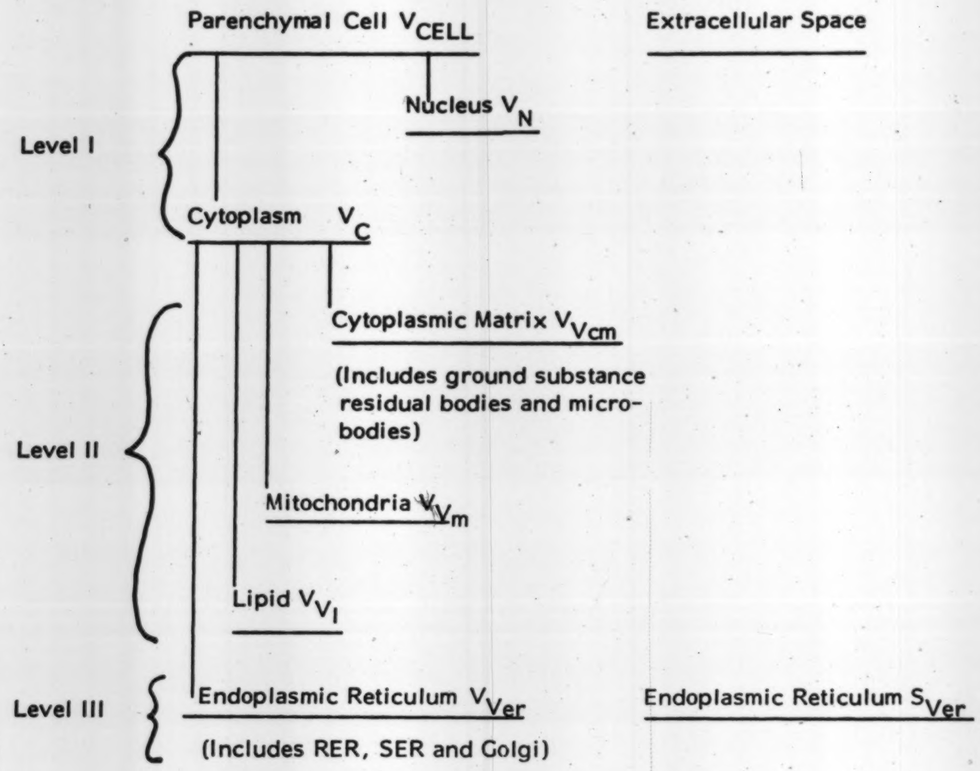
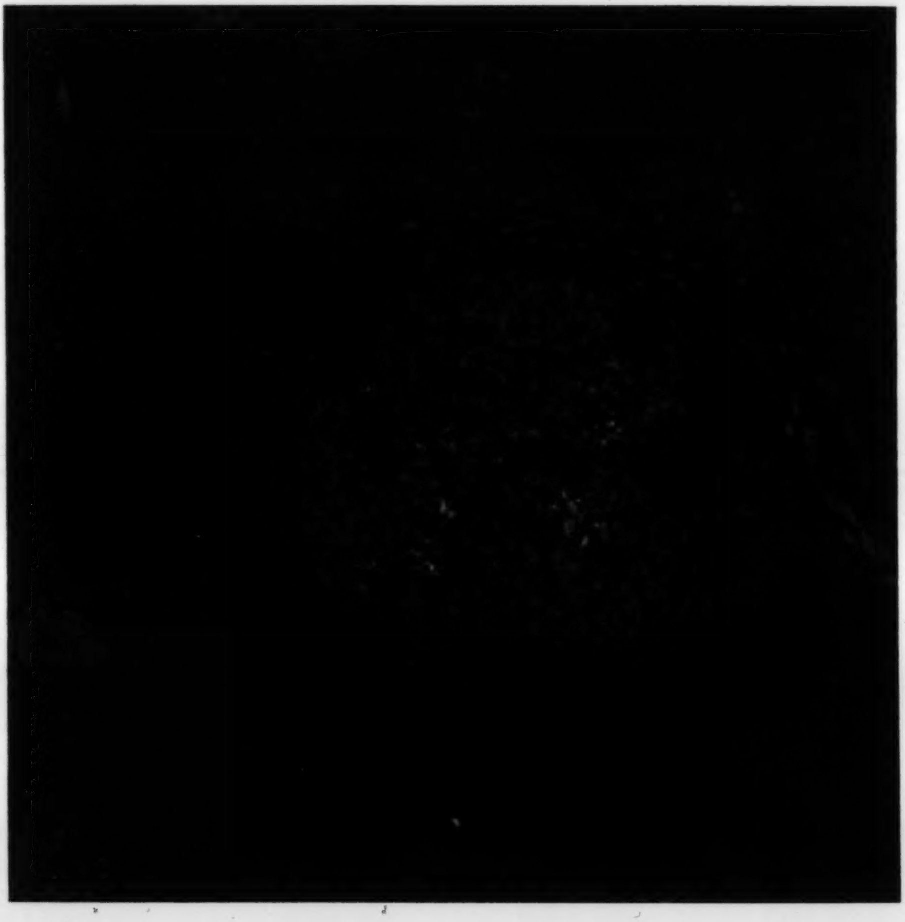

Parenchymal Cell V_{CELL}
Extracellular Space
Nucleus V_N
Level I
Cytoplasm V_C
Cytoplasmic Matrix $V_{V_{cm}}$
(Includes ground substance residual bodies and microbodies)
Level II
Mitochondria V_{V_m}
Lipid V_{V_l}
Level III
Endoplasmic Reticulum $V_{V_{er}}$
Endoplasmic Reticulum $S_{V_{er}}$
(Includes RER, SER and Golgi)

Figure 7. The zona fasciculata of the rat one day after hypophysectomy.

15,000X



XUM

Figure 8. A higher magnification of the zona fasciculata of the rat one day after hypophysectomy showing the presence of a dense granule within a mitochondrion (arrow) and an increased incidence of tubular cristae appearing in some mitochondria. 46,000X

XUM

Figure 9. The zona fasciculata of the rat two days after hypophysectomy. The arrows point out what appears to be two unmyelinated nerve fibers. 15,000X

XUM

Figure 10. The zona fasciculata of the rat two days after hypophysectomy. Note the cell in the upper right hand corner possessing mitochondria containing vesiculo-tubular or tubular cristae and the cell in the left side of the field which might be the precursor of a blood cell. 14,000X

XUM

XUM

Figure 11. A higher magnification of the zona fasciculata two days after hypophysectomy. Note the tubular shape of the SER, the two small residual bodies, and the intramitochondrial tubules (arrow). 46,000X.

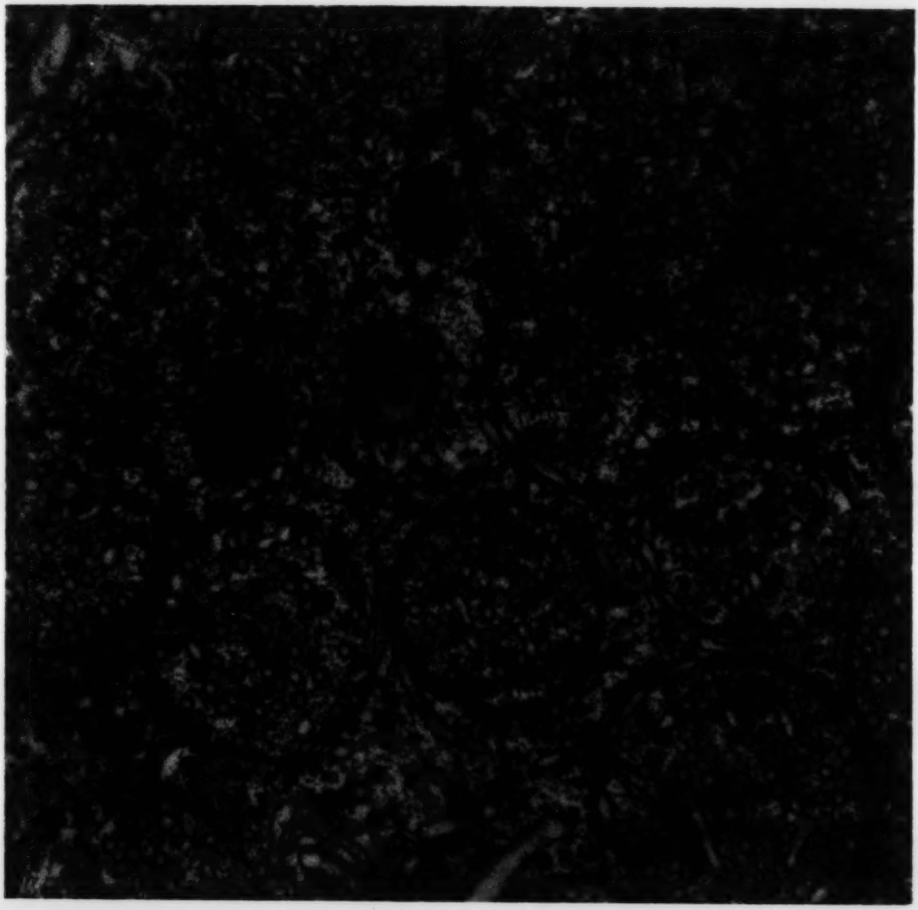
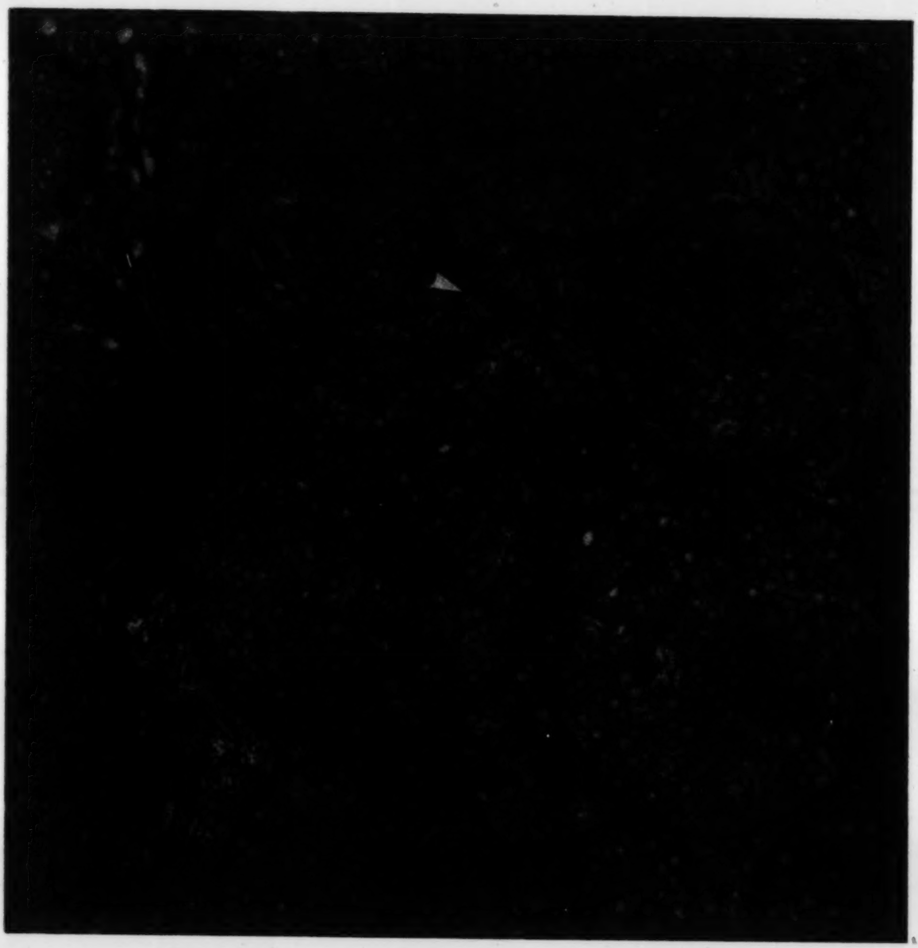


Figure 12. The zona fasciculata of the rat four days after hypophysectomy. Note the great variation in the size of the mitochondria. 15,000X.

XUM

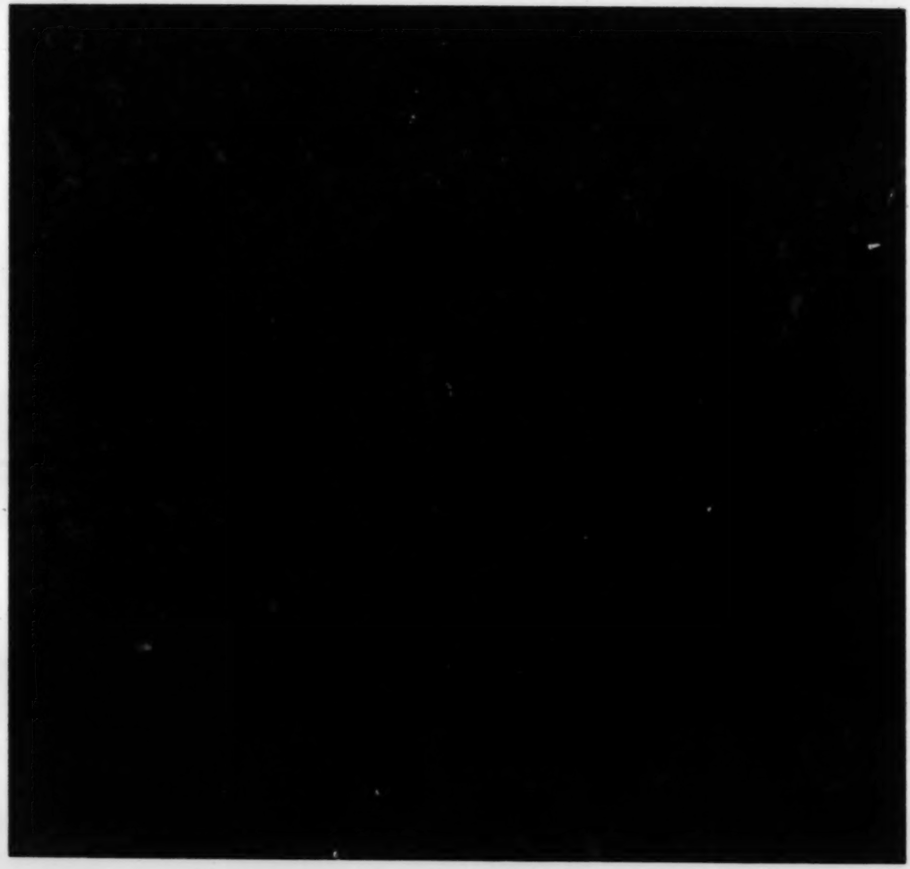
Figure 13. A higher magnification of the zona fasciculata four days after hypophysectomy. The arrow points out a bit of the scarce RER. 46,000X.

XUM



XUM

Figure 14. The zona fasciculata of the rat ten days after hypophysectomy. Most nuclei are no longer spherical at this time. 14,000X.



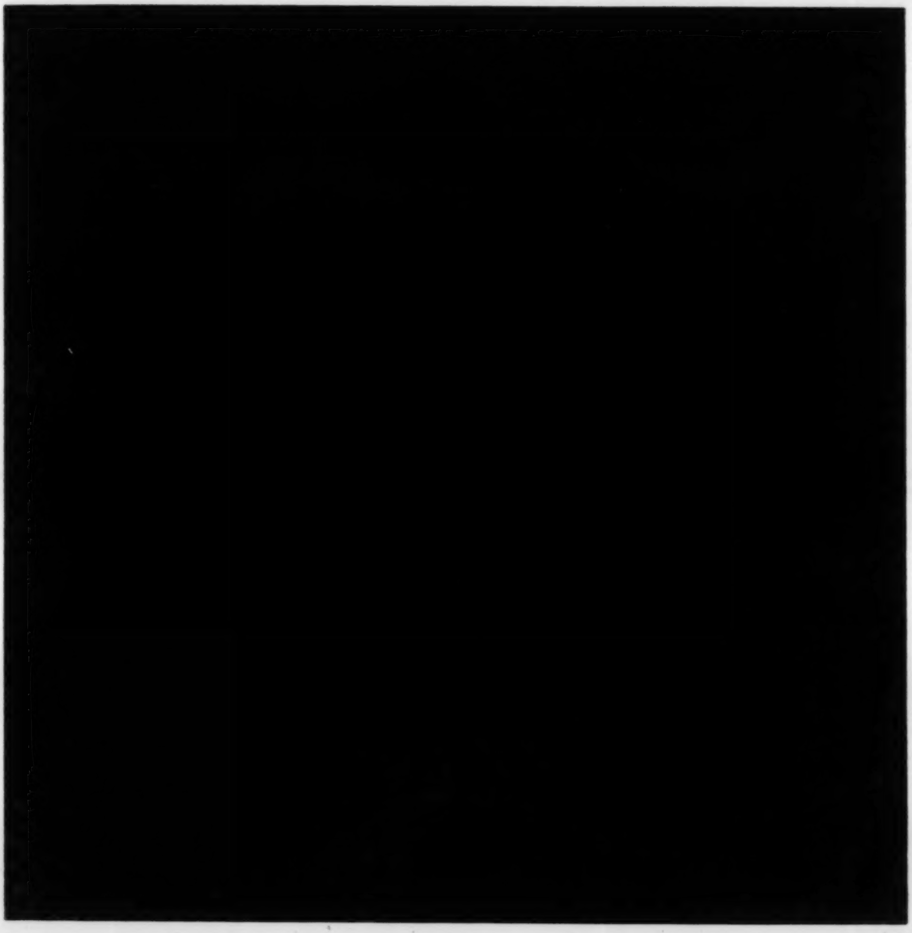
XUM

Figure 15. The zona fasciculata of the rat ten days after hypophysectomy. This is an illustration of the great accumulation of lipid droplets occurring in parenchymal cells as well as other cell types (arrows) at this time. 14,000X.

XUM

Figure 16. A higher magnification of the zona fasciculata ten days after hypophysectomy. Note the irregular mitochondria containing vesiculo-tubular cristae. Notice, also, the still quite numerous profiles of SER. 46,000X.

XUM

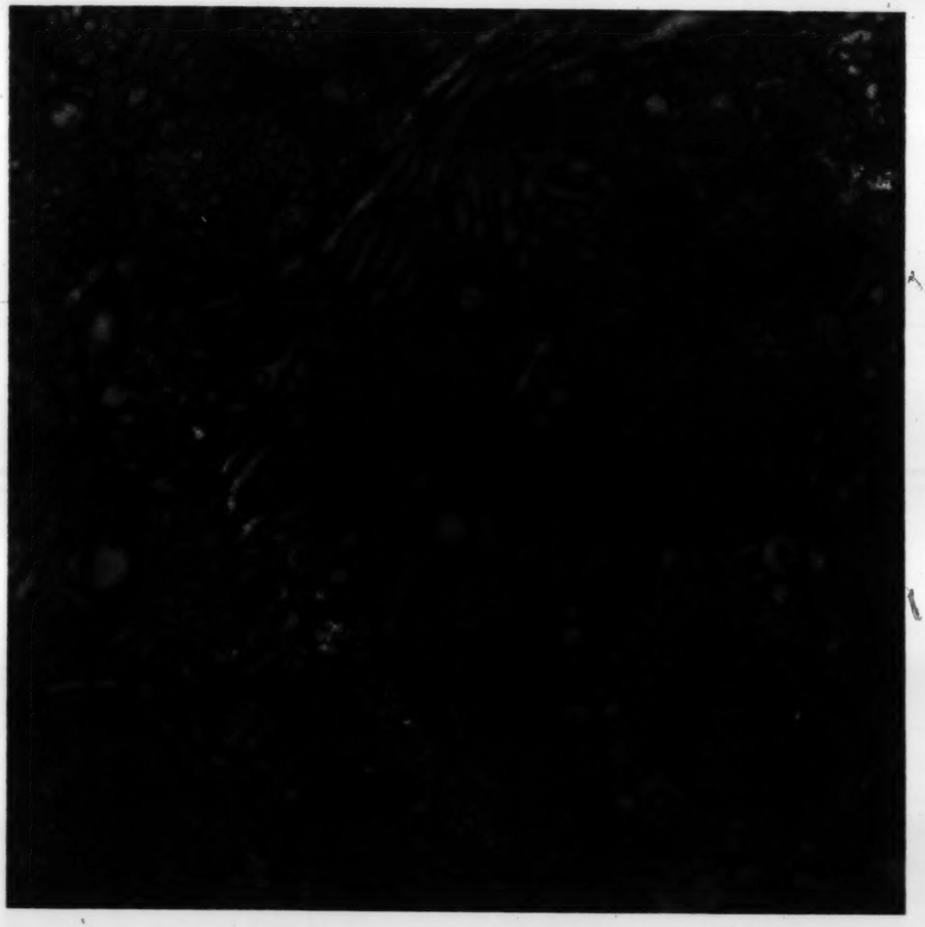


XUM

Figure 17. A higher magnification micrograph of the zona fasciculata of the rat ten days after hypophysectomy. 46,000X.

XUM

Figure 18. The zona fasciculata of the rat ten days after hypophysectomy. Notice the unusual arragement of the SER. 44,000X.



XUM

Figure 19. The zona fasciculata of the rat fourteen days after hypophysectomy. Note the enormous accumulation of lipid droplets and the small size of the mitochondria containing mostly vesicular cristae. 14,000X.

XUM

Figure 20. A low magnification micrograph of the zona fasciculata of the rat fourteen days after hypophysectomy. Almost the entire field is occupied by one parenchymal cell. Note the small irregularly shaped mitochondria possessing vesicular or vesiculo-tubular cristae. 15,000X.

XUM

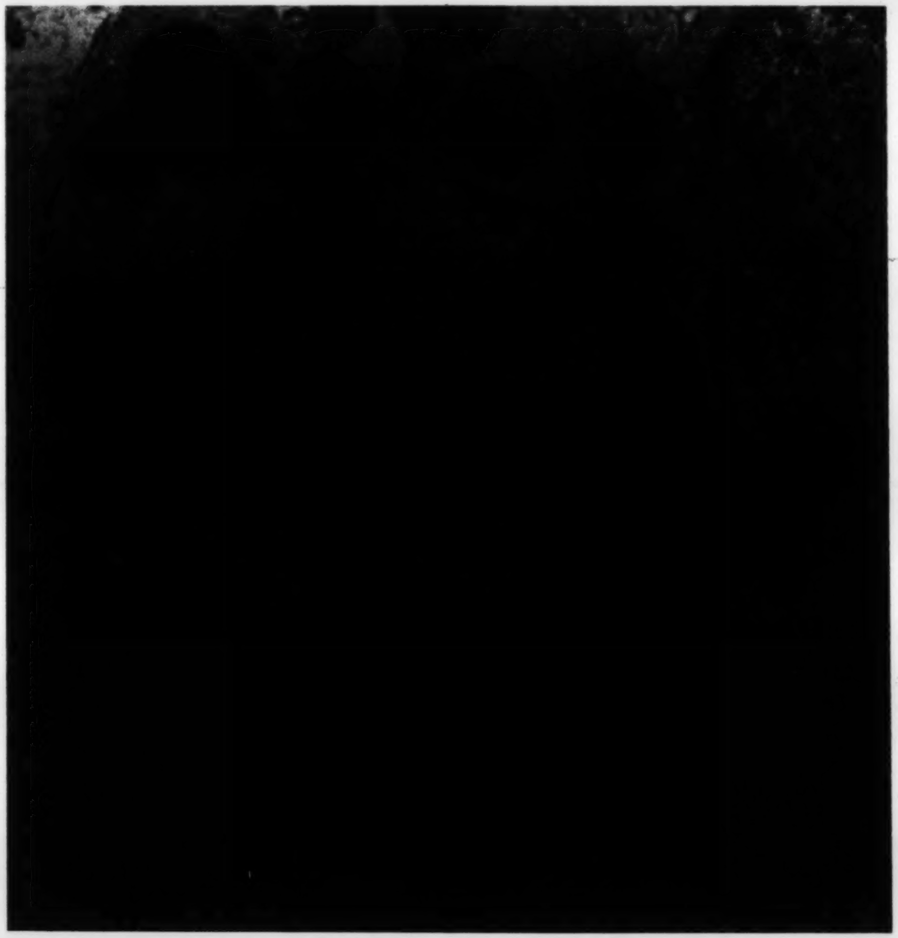
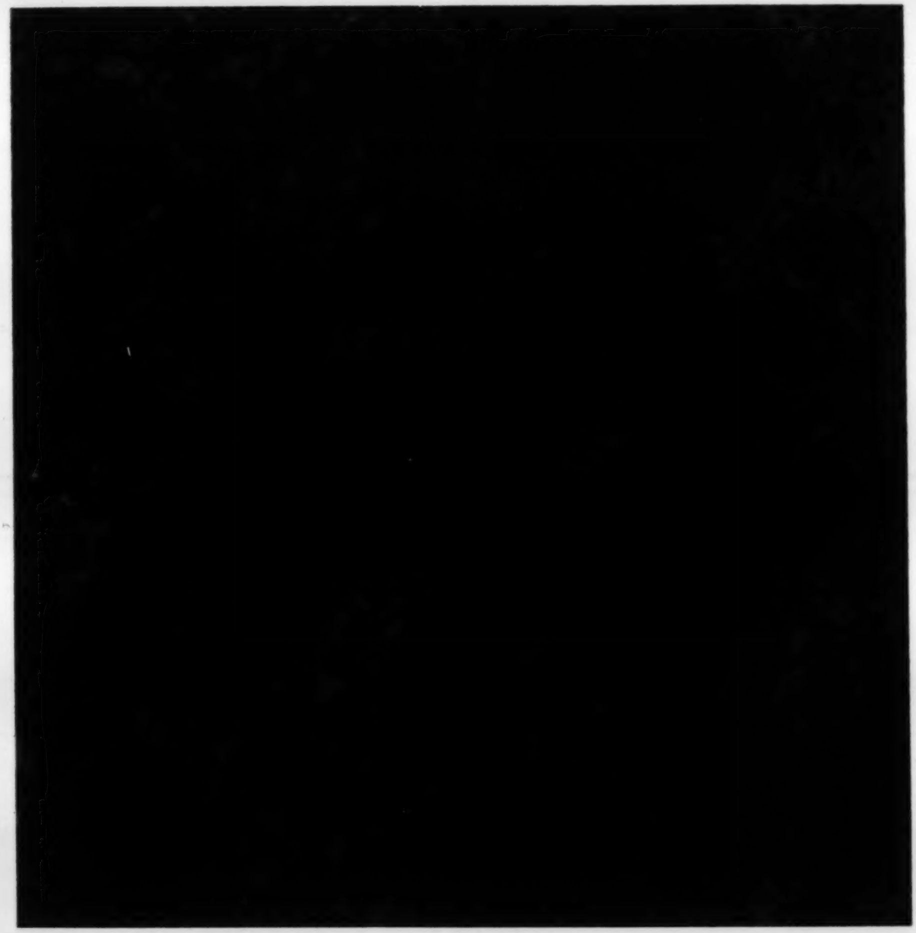


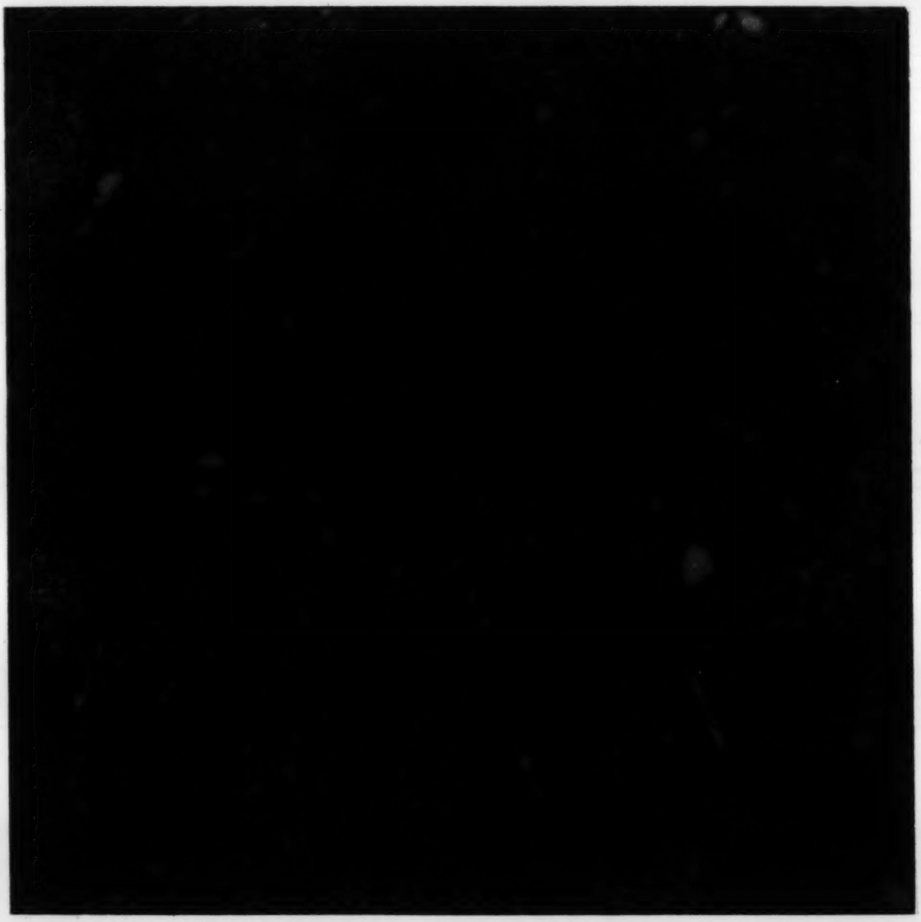
Figure 21. A high magnification micrograph of the zona fasciculata fourteen days after hypophysectomy. Note that the SER is poorly developed. 46,000X.

XUM



XUM

Figure 22. A high magnification micrograph of the zona fasciculata fourteen days after hypophysectomy. This micrograph is the same magnification as that of Figure 21. Observe the huge quantity of SER seemingly flowing through the cytoplasm. 46,000X.



XUM

Figure 23. A graph of the relative volumes of the various parameters plotted against time after hypophysectomy.

XUM

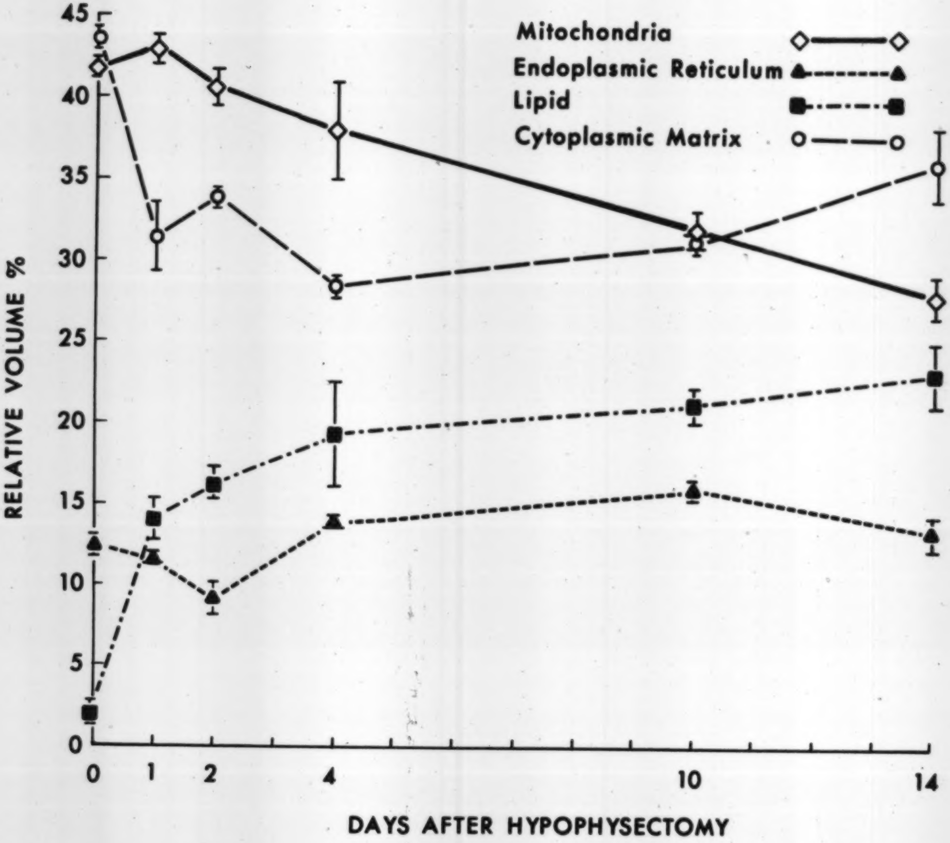

Mitochondria ◇——◇
Endoplasmic Reticulum ▲-------▲
Lipid ■-·-·-■
Cytoplasmic Matrix ○— —○
RELATIVE VOLUME %
45
40
35
30
25
20
15
10
5
0
0
1
2
4
10
14
DAYS AFTER HYPOPHYSECTOMY

Figure 24. A graph showing the decline in absolute volume of the fasciculata parenchymal cell after hypophysectomy.

XUM

XUM

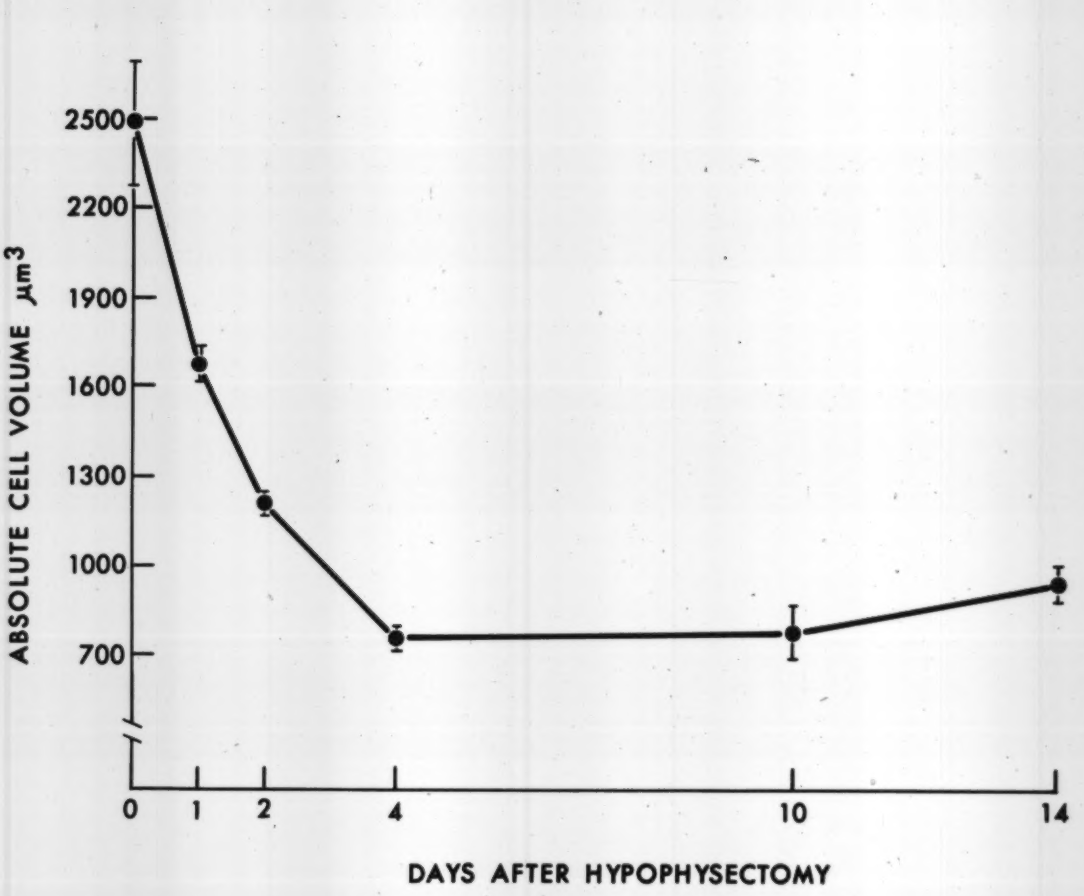

ABSOLUTE CELL VOLUME μm^3
2500
2200
1900
1600
1300
1000
700
0
1
2
4
10
14
DAYS AFTER HYPOPHYSECTOMY

Figure 25. A graph illustrating how the corticosterone plasma levels and the absolute volumes of mitochondria and lipid vary with time after hypophysectomy.

XUM

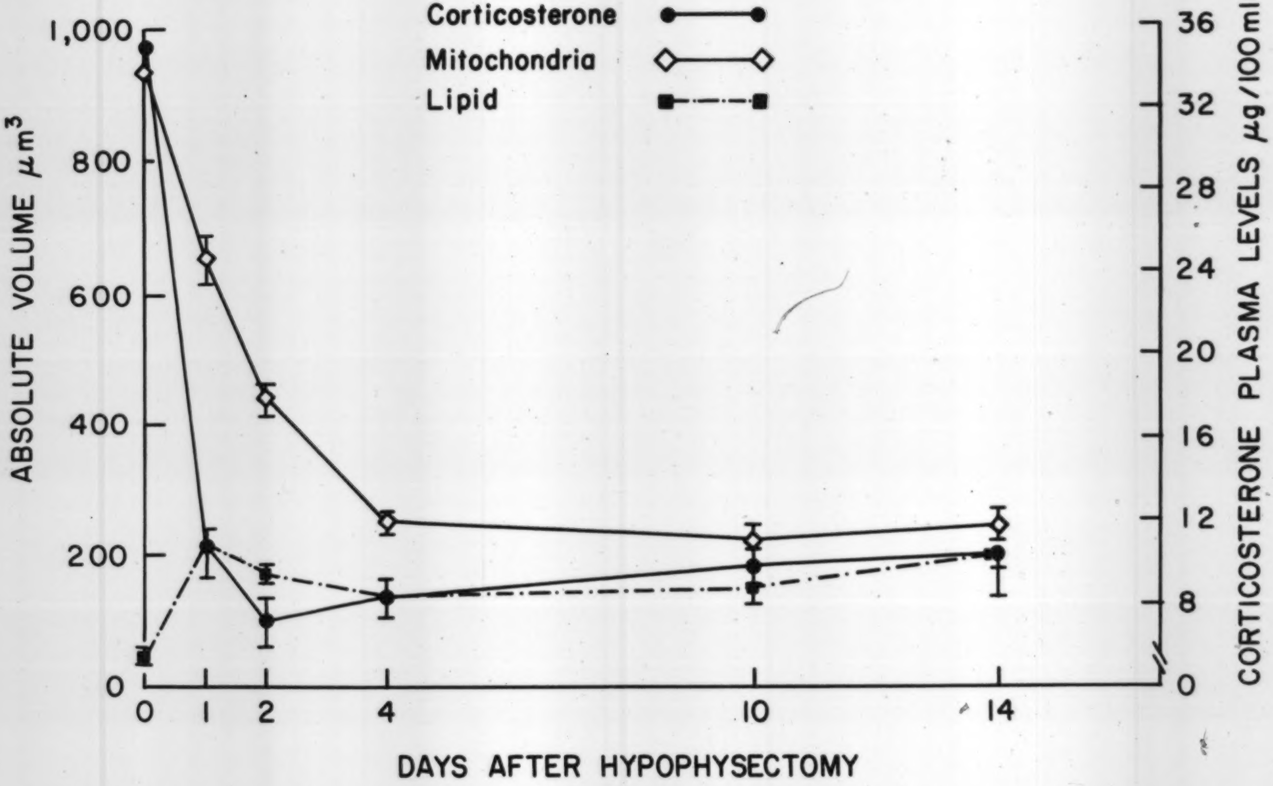

Corticosterone
Mitochondria
Lipid
ABSOLUTE VOLUME μm^3
1,000
800
600
400
200
0
CORTICOSTERONE PLASMA LEVELS μg/100ml
36
32
28
24
20
16
12
8
0
0
1
2
4
10
14
DAYS AFTER HYPOPHYSECTOMY

Figure 26. A graph illustrating how the corticosterone plasma levels and the absolute volumes of the cytoplasmic matrix and ER vary with time after hypophysectomy.

XUM

XUM

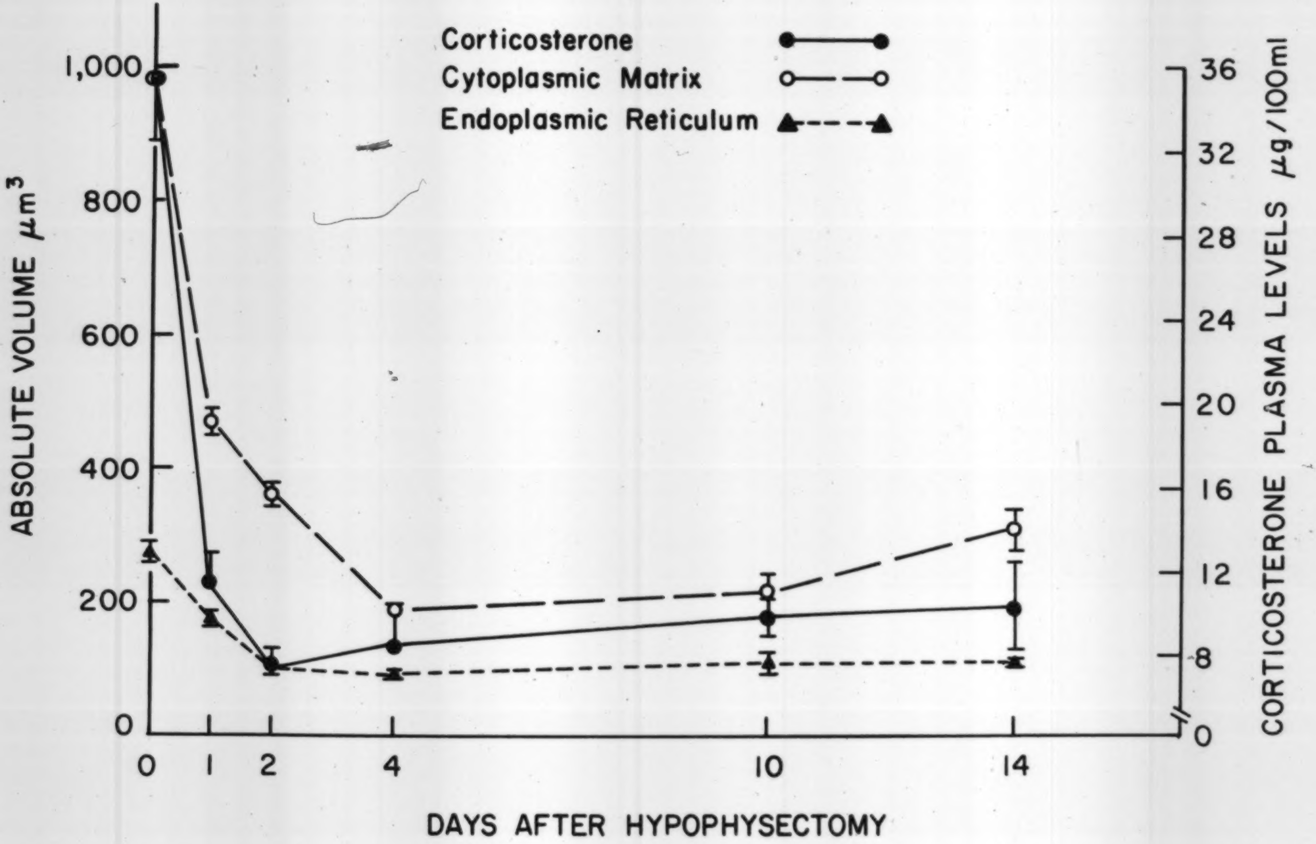

Corticosterone ●—●
Cytoplasmic Matrix ○— —○
Endoplasmic Reticulum ▲----▲
ABSOLUTE VOLUME μm^3
1,000
800
600
400
200
0
0
1
2
4
10
14
DAYS AFTER HYPOPHYSECTOMY
36
32
28
24
20
16
12
8
0
CORTICOSTERONE PLASMA LEVELS $\mu g/100ml$

Figure 27. A graph comparing the decline in corticosterone plasma levels and the surface density of the ER with time after hypophysectomy.

XUM

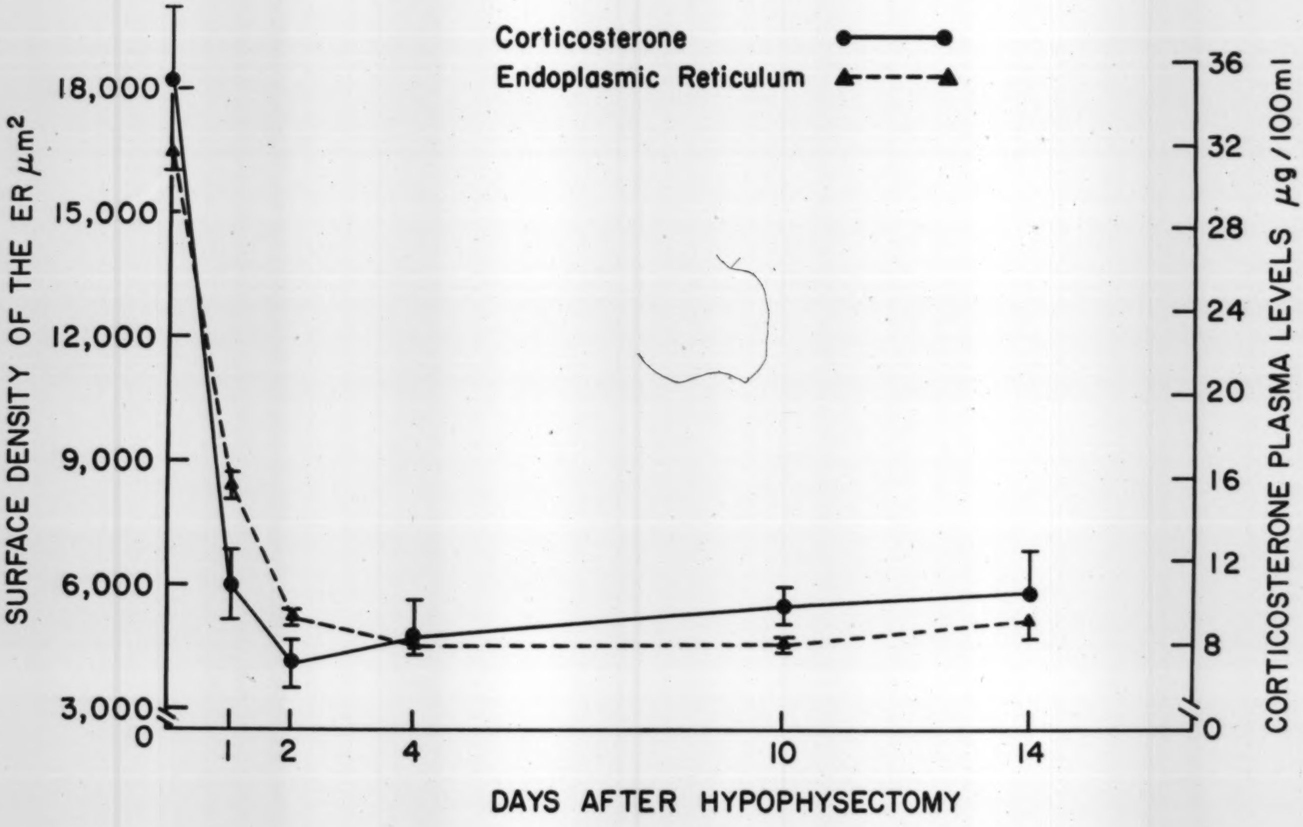

Corticosterone
Endoplasmic Reticulum
SURFACE DENSITY OF THE ER μm^2
18,000
15,000
12,000
9,000
6,000
3,000
0
1
2
4
10
14
DAYS AFTER HYPOPHYSECTOMY
CORTICOSTERONE PLASMA LEVELS μg/100ml
36
32
28
24
20
16
12
8
0

Figure 28. The zona intermedia of the rat four days after hypophysectomy. This area borders on the zona glomerulosa and illustrates mitochondria that resemble those of the glomerulosa. 15,000X.

XUM

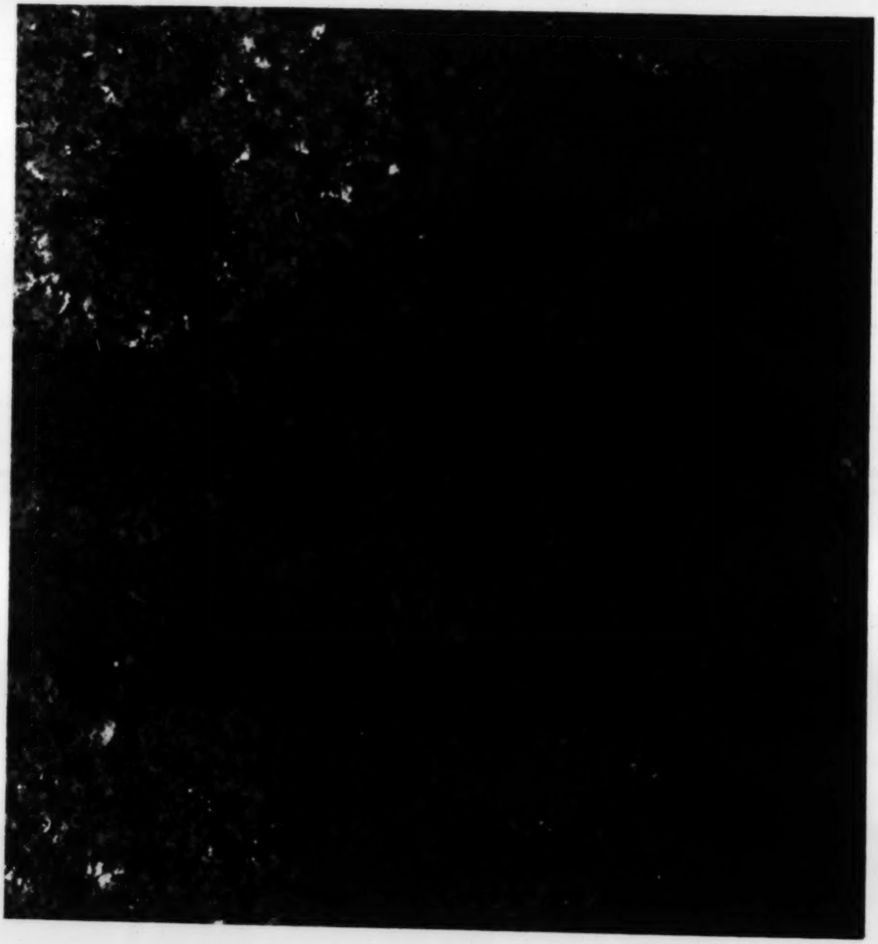


Figure 29. A higher magnification of the zona intermedia of the rat four days after hypophysectomy. 50,000X.

XUM

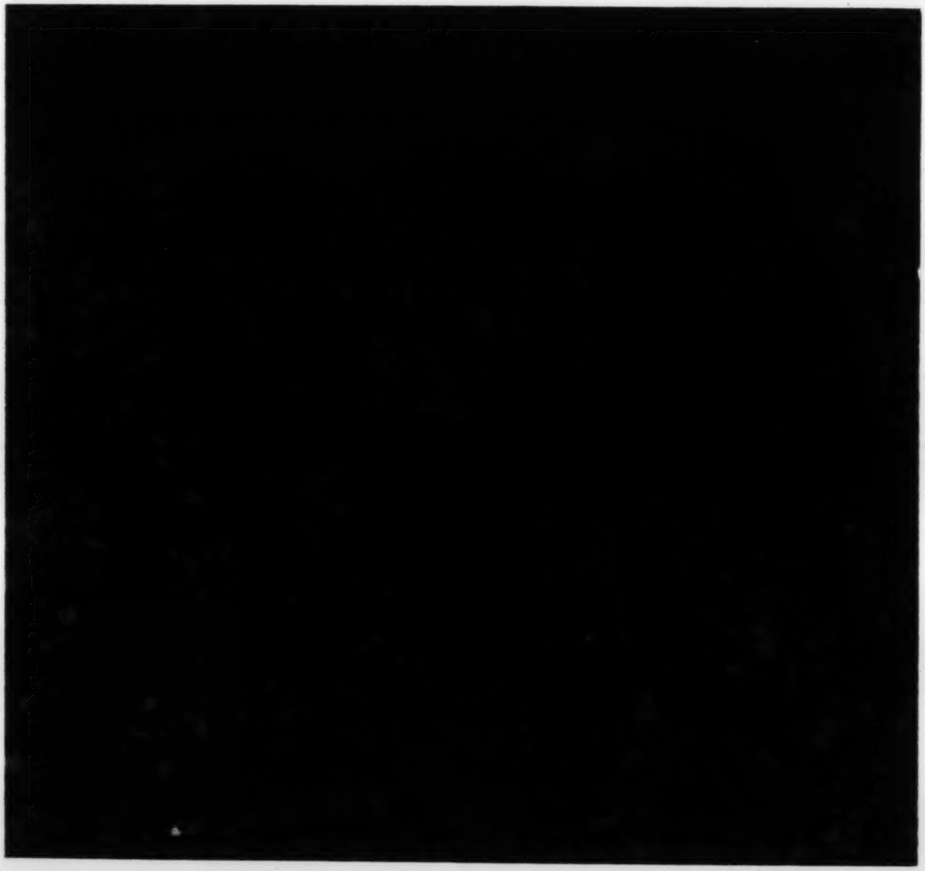
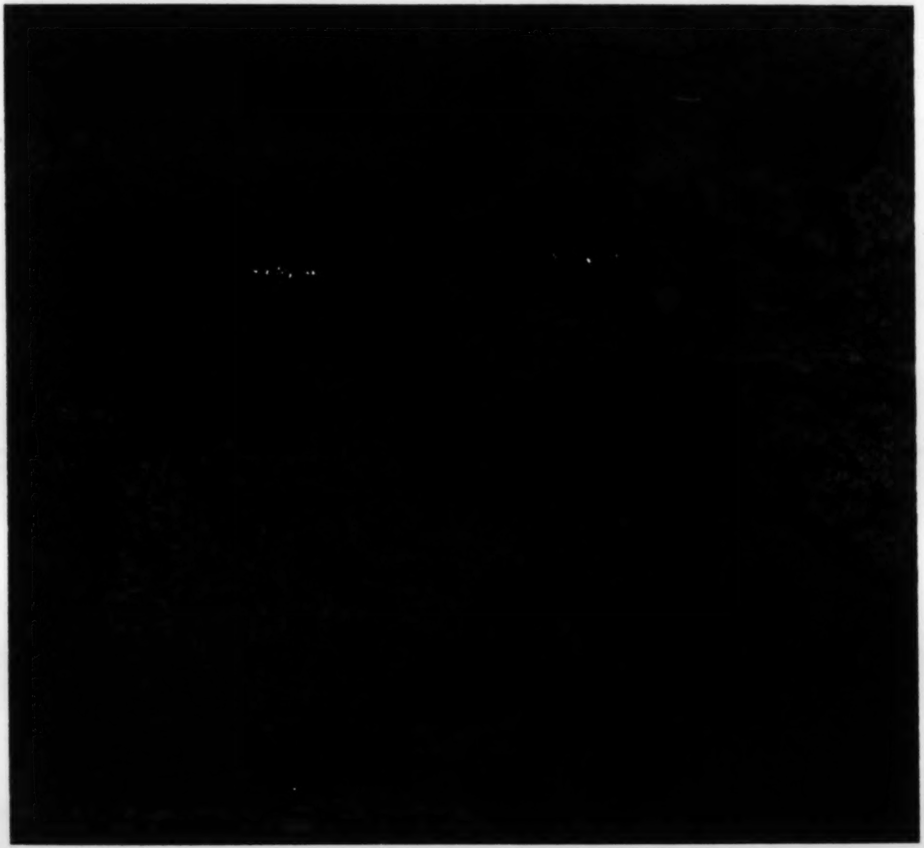


Figure 30. The zona intermedia of the rat four days after hypophysectomy. This area borders on the zona fasciculata and illustrates mitochondria that resemble those of the fasciculata of the intact rat. 14,000X.



XUM

Figure 31. A higher magnification of the zona intermedia of the rat four days after hypophysectomy. This area borders on the zona fasciculata and illustrates mitochondria that resemble those of the fasciculata of the intact rat. 48,000X.

XUM

Figure 32. The zona intermedia of the rat ten days after hypophysectomy. This area borders on the zona glomerulosa and illustrates mitochondria that resemble those of the glomerulosa of the intact rat. 20,000X

XUM

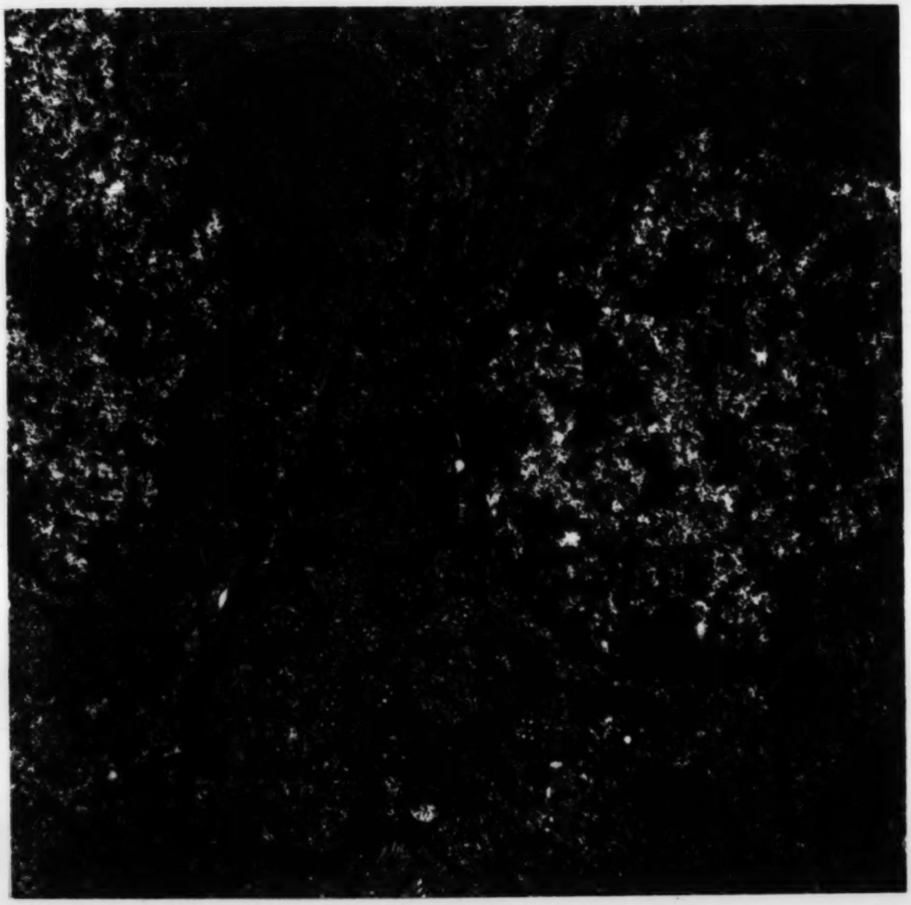
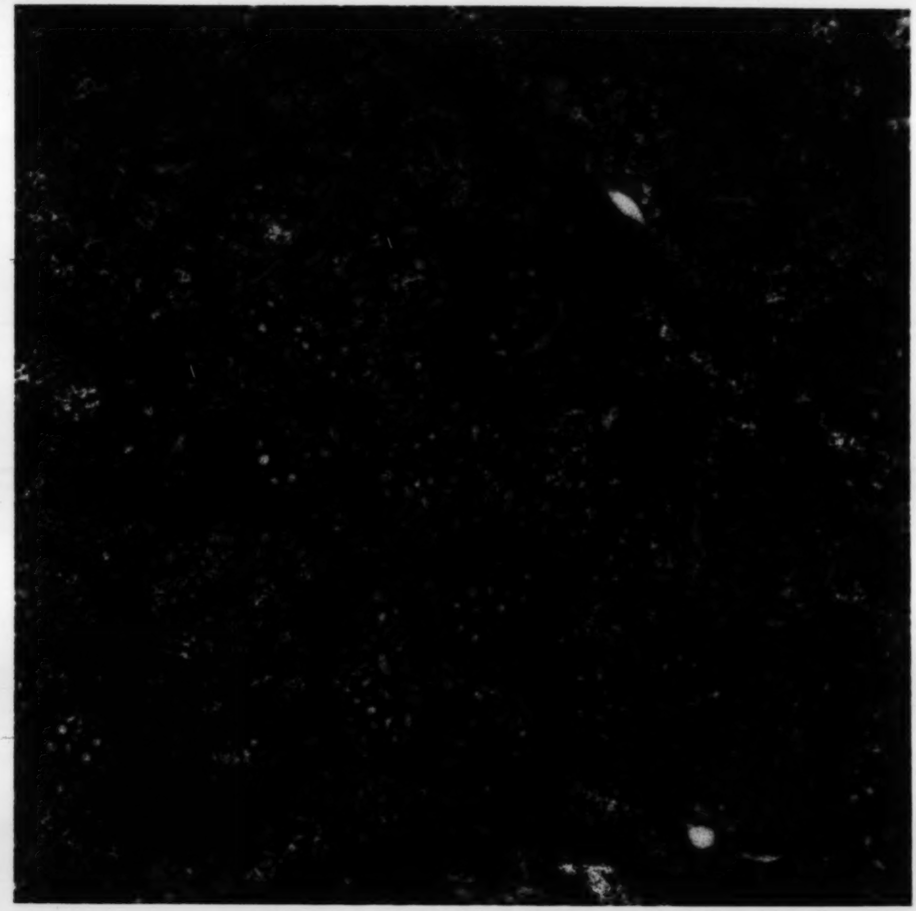


Figure 33. A higher magnification of the zona intermedia of the rat ten days after hypophysectomy. This area is from the same field as Figure 32. 46,000X.

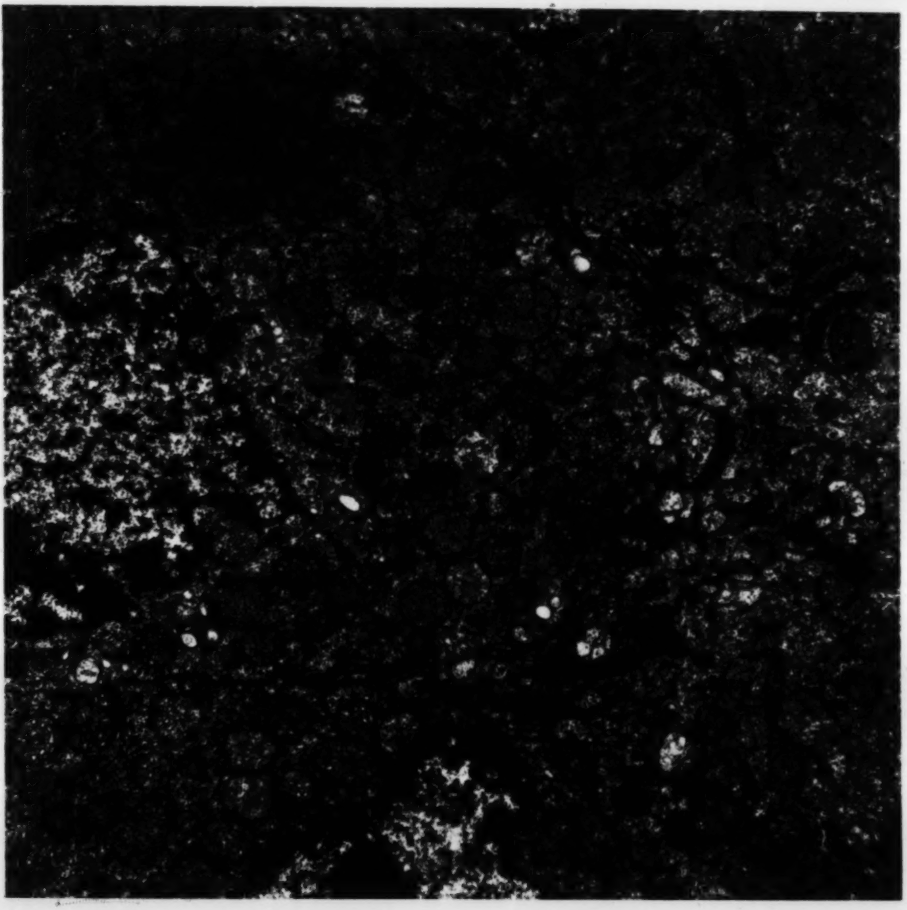
XUM



XUM

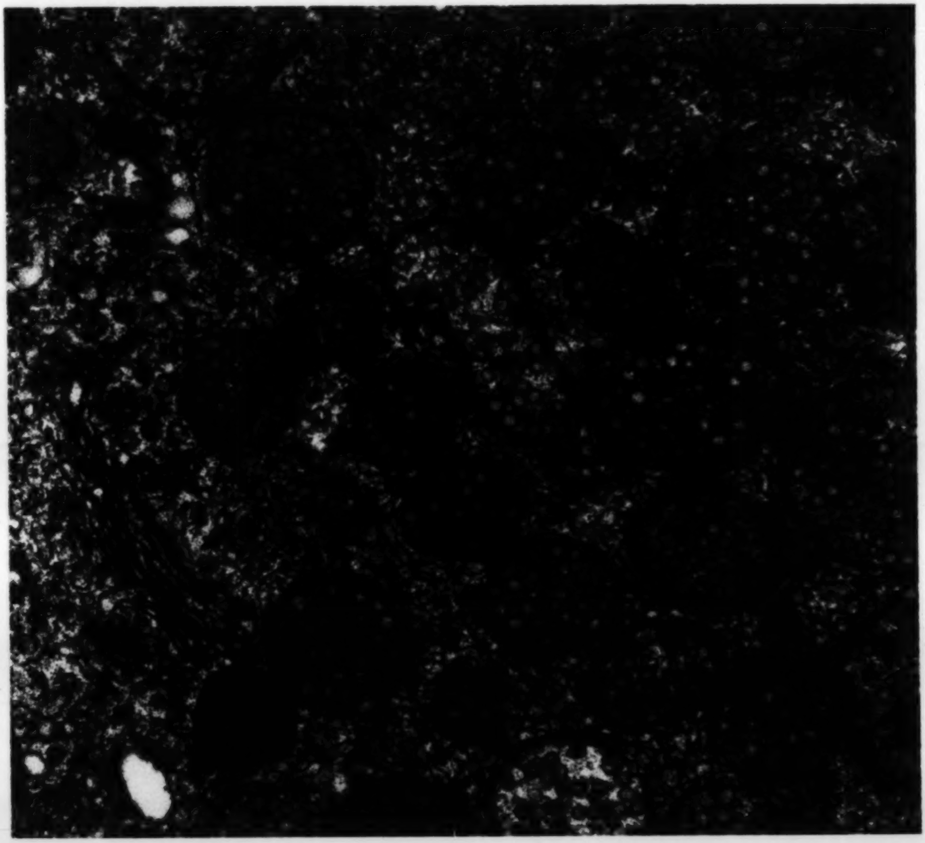
Figure 34. The zona intermedia of the rat ten days after hypophysectomy. This area borders on the zona fasciculata and illustrates mitochondria that resemble those of the fasciculata of the intact rat. 15,000X.

XUM



XUM

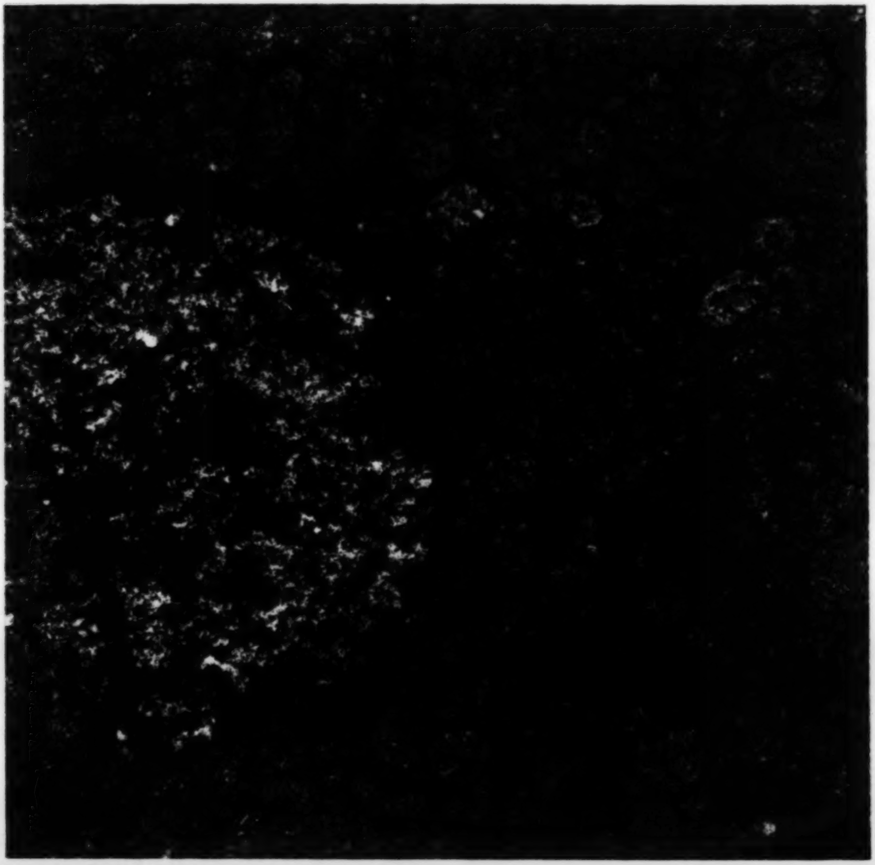
Figure 35. A higher magnification of the zona intermedia of the rat ten days after hypophysectomy. This area is from the same field as Figure 34. 46,000X.



XUM

Figure 36. The zona intermedia of the rat ten days after hypophysectomy. This field was taken from the middle of zone and illustrates the two different cell types discussed in the text existing side by side. The cell on the left possesses mitochondria resembling those of glomerulosa cells from intact rats and the cell on the right possesses mitochondria resembling those of fasciculata cells from intact rats. 20,000X.

XUM



XUM

Figure 37. A higher magnification of the zona intermedia taken from the same field as Figure 36. 44,000X.

XUM

XUM

Figure 38. The zona intermedia of the rat fourteen days after hypophysectomy. This area is taken from the middle of the zone to illustrate the diversity of mitochondrial types present. The arrow points out a branched mitochondrion. 14,000X.

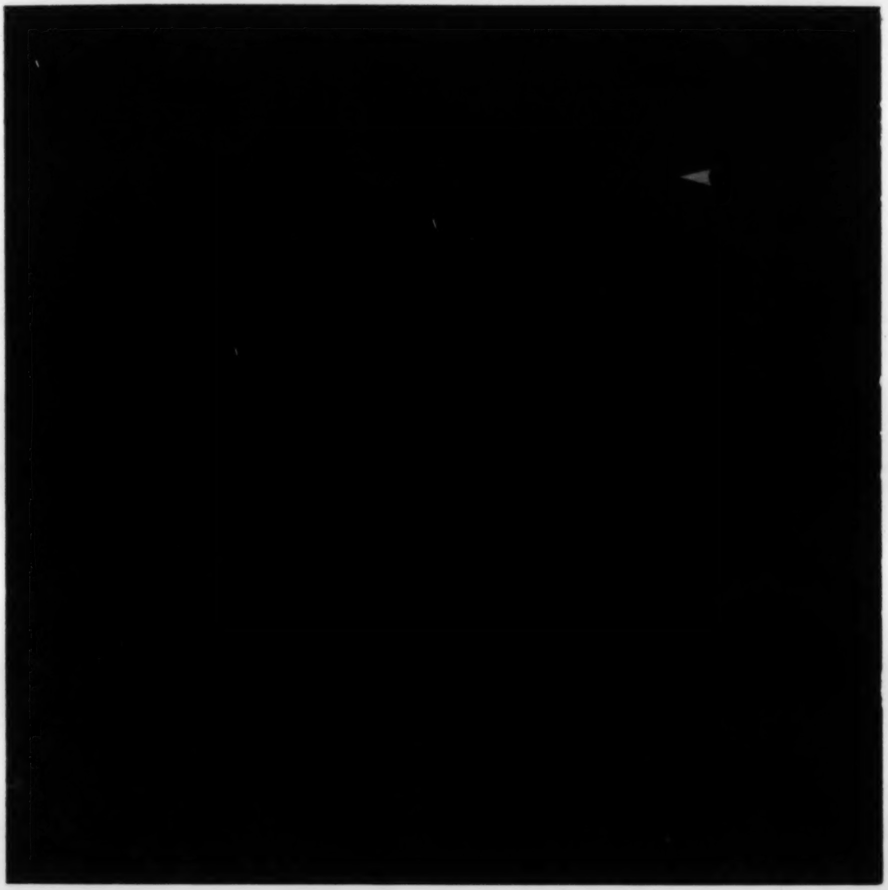
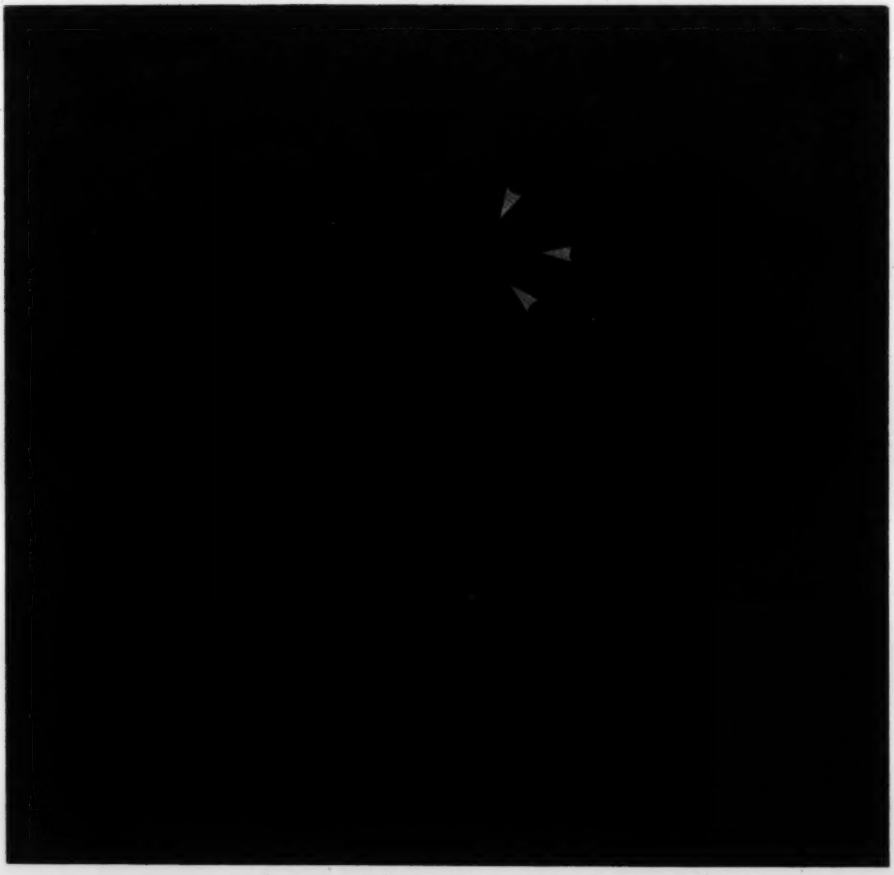


Figure 39. A higher magnification of the zona intermedia of the rat fourteen days after hypophysectomy taken from the same field as Figure 38. The arrows point out the moderate amount of SER present, a clump of glycogen particles, and the presence of dense granules within some of the mitochondria. 44,000X.



XUM

TABLE 1: Relative Volumes of Morphometric Parameters of Fasciculata Parenchymal Cytoplasm

Days after hypophysectomy		Control	1 day	2 days	4 days	10 days	14 days
Volume of mitochondria	%	41.8	42.9	40.6	38.2	31.9	27.7
	S.E.M.	0.5	0.7	1.1	2.9	1.1	1.3
	Δ %		+3	-3	-9	-24	-34
	P		N.S.	N.S.	N.S.	<0.005	<0.005
Volume of lipid	%	2.1	14.2	16.1	19.6	21.1	23.1
	S.E.M.	0.5	1.3	0.7	3.3	1.0	2.0
	Δ %		+576	+667	+883	+905	+1000
	P		<0.005	<0.005	<0.005	<0.005	<0.005
Volume of endoplasmic reticulum	%	12.4	11.5	9.4	13.9	15.8	13.3
	S.E.M.	0.6	0.3	0.8	0.3	0.6	0.9
	Δ %		-7	-24	+12	+27	+7
	P		N.S.	<0.01	<0.05	<0.005	N.S.
Volume of cytoplasmic matrix	%	43.7	31.4	33.9	28.3	31.1	35.9
	S.E.M.	0.8	1.8	0.6	0.5	0.6	2.4
	Δ %		-28	-22	-35	-29	-18
	P		<0.005	<0.005	<0.005	<0.005	<0.01
Volume of nucleus	%	9.0	9.7	11.5	11.4	9.1	7.3
	S.E.M.	0.5	0.5	0.4	0.4	0.8	0.7
	Δ %		+8	+28	+27	+1	-19
	P		N.S.	<0.005	<0.005	N.S.	<0.05
Volume of cytoplasm	%	91.0	90.3	88.3	88.6	90.9	92.7
	S.E.M.	0.5	0.5	0.4	0.4	0.8	0.7
	Δ %		-1	-3	-3	0	+2
	P		N.S.	<0.005	<0.005	N.S.	<0.05

*The values for the relative volumes of the mitochondria, lipid, endoplasmic reticulum and cytoplasmic matrix are given in relation to the cytoplasmic volume. N.S. = not significant (P >0.05).

XUM

XUM

TABLE 2: Corticosterone Plasma Levels*

Days after hypophysectomy	Control	1 day	2 days	4 days	10 days	14 days
Plasma Levels µg/100 ml.	35.5	11.0	7.2	8.4	9.8	10.4
S.E.M.	3.5	1.7	1.1	1.8	0.9	2.1
Δ %		-69	-80	-76	-72	-71
P		<0.005	<0.005	<0.005	<0.005	<0.005

*The value for each time period is a mean of 4 animals.

TABLE 3: Absolute Volumes of Morphometric Parameters of Fasciculata Parenchymal Cytoplasm.

Days after hypophysectomy		Control	1 day	2 days	4 days	10 days	14 days
Volume of cell	μm^3	2465.8	1674.7	1213.1	756.1	767.5	940.0
	S.E.M.	218.8	60.4	37.7	39.8	94.0	63.4
	Δ %		-32	-51	-69	-69	-62
	P		<0.005	<0.005	<0.005	<0.005	<0.005
Volumes of nucleus	μm^3	216.2	161.3	139.0	85.7	66.7	66.9
	S.E.M.	7.6	5.0	5.6	2.9	2.8	2.4
	Δ %		-25	-36	-60	-69	-69
	P		<0.005	<0.005	<0.005	<0.005	<0.005
Volume of cytoplasm	μm^3	2249.6	1513.4	1074.0	670.4	700.9	873.0
	S.E.M.	211.7	62.3	35.2	37.3	91.5	64.7
	Δ %		-33	-52	-70	-69	-61
	P		<0.01	<0.005	<0.005	<0.005	<0.005
Volume of mitochondria	μm^3	943.9	650.8	436.7	253.2	220.8	242.6
	S.E.M.	95.3	36.2	22.3	15.3	24.2	23.7
	Δ %		-31	-54	-73	-77	-74
	P		<0.01	<0.005	< 0.005	<0.005	<0.005
Volume of lipid	μm^3	48.9	216.3	173.4	135.1	150.8	202.0
	S.E.M.	12.3	23.0	9.8	29.3	26.5	21.2
	Δ %		+342	+255	+176	+208	+313
	P		<0.005	<0.005	<0.01	<0.005	<0.005
Volume of endoplasmic reticulum	μm^3	275.4	175.3	100.1	92.9	111.9	114.1
	S.E.M.	19.8	11.3	5.3	4.9	16.3	6.7
	Δ %		-36	-64	-66	-59	-59
	P		<0.005	<0.005	<0.005	<0.005	<0.005
Volume of cytoplasmic matrix	μm^3	981.5	471.3	363.9	189.3	217.5	314.2
	S.E.M.	94.4	17.8	16.0	8.8	26.8	32.4
	Δ %		-52	-63	-81	-78	-68
	P		<0.005	<0.005	<0.005	<0.005	<0.005
Surface density of endoplasmic reticulum	μm^2	16,437.4	8401.8	5229.8	4458.0	4466.5	4951.4
	S.E.M.	398.3	340.7	210.9	189.5	131.5	415.9
	Δ %		-49	-68	-73	-73	-70
	P		<0.005	<0.005	<0.005	<0.005	<0.005

*The values for the absolute volumes of the mitochondria, lipid, endoplasmic reticulum, cytoplasmic matrix and surface density of the ER are given in relation to the cytoplasmic volume.

XUM

LITERATURE CITED

Ashmore, J. and D. Morgan, 1967 Metabolic effects of adrenal glucocorticoid hormones. In The Adrenal Cortex. (ed. A.B. Eisenstein). Little, Brown and Co., Boston. pp. 249-267.

Borowicz, J.W., 1965 Some ultrastructural changes in adrenal cortical cells of rat after hypophysectomy and following ACTH administration. Beit. Path. Anat. Allg-Path. 132: 441-468.

Bush, I.E., 1953 Species differences in adrenocortical secretion. J. Endocrinol. 9: 95-100.

Canick, J.A. and J.L. Purvis, 1972 The maintenance of mitochondrial size in the rat adrenal cortex zona fasciculata by ACTH. Exp. Mol. Path. 16: 79-93.

Cater, D.B. and J.D. Lever, 1954 The zona intermedia of the adrenal cortex. A correlation of possible functional significance with development, morphology and histochemistry. J. Anat. 88: 437-454.

Cater, D.B. and M.P. Stack-Dunne, 1953 The histological changes in the adrenal of the hypophysectomized rat after treatment with pituitary preparations. J. Path. Bact. 66: 119-133.

Christensen, A.K. and S.W. Gillim, 1969 The correlation of fine structure and function in steroid-secreting cells with emphasis on those of the gonads. In The Gonads (ed. K.W. McKerns). Appleton-Century-Crofts, New York. pp. 415-488.

Colby, H.D., L.K. Malendowicz, J.L. Caffrey and J.I. Kitay, 1974 Effects of hypophysectomy and ACTH on adrenocortical function in the rat. Endocrinol. 94: 1346-1350.

XUM

Dalton, A.J., E.R. Mitchell, B.F. Jones and V.B. Peters, 1944 Changes in the adrenal glands of rats following exposure to lowered oxygen tension. J. Nat. Cancer Inst. 4: 527-536.

Deane, H.W., 1958 Intracellular lipides: their detection and significance. In Frontiers in Cytology (ed. S.L. Palay). Yale University Press, New Haven, pp. 227-263.

Deane, H.W., 1962 The anatomy, chemistry, and physiology of adrenocortical tissue. In Handbuch Der Experimentellen Pharmakologie (ed. H. W. Deane), 14 (1). Springer-Verlag, Berlin. p. 1-185.

Deane, H.W. and R.O. Greep, 1946 A morphological and histochemical study of the rat's adrenal cortex after hypophysectomy, with comments on the liver. An. J. Anat. 79: 117-145.

Deane, H.W., J.H. Shaw and R.O. Greep, 1948 The effect of altered sodium or potassium intake on the width and cytochemistry of the zona glomerulosa of the rat's adrenal cortex. Endocrinol. 43: 133-153.

deGroot, J. and C. Fortier, 1959 Quantitative and histological aspects of adrenal cortical regeneration in the male albino rat. Anat. Rec. 133: 565-577.

Delesse, M.A., 1848 Procédé mécanique pour déterminer la composition des roches. Ann. Mines. 13: 379-388.

Ebbesson, S.O. and D.B. Tang, 1967 A comparison of sampling procedures in a structured cell population. In Stereology(ed. H. Elias). Springer-Verlag Berlin, Heidelberg, New York. pp. 131-132.

XUM

Farese, R.V., 1968 Regulation of adrenal growth and steroidogenesis by ACTH. In Functions of the Adrenal Cortex (ed. K.W. McKerns). Vol. 1. Appleton-Century-Crofts, New York. pp. 539-581.

Ford, J.K. and R.W. Young, 1963 Cell proliferation and displacement in the adrenal cortex of young rats injected with tritiated thymidine. Anat. Rec. 146: 125-137.

Fujita, H., 1972 On the fine structure of alteration of the adrenal cortex in hypophysectomized rats. Z. Zellforsch. 125: 480-496.

Ganong, W.F., 1963 The central nervous system and the synthesis and release of ACTH. In Advances in Neuroendocrinology (ed. A.V. Nalbandov). University of Illinois Press, Urbana. pp. 92-149.

Ganong, W.F. and E.E. Van Brunt, 1968 Control of aldosterone secretion In Handbuch der Experimentellen Pharmakologie (ed. H.W. Deane and B.L. Rubin) 14(3). Springer-Verlag, Berlin, Heidelberg, New York. pp. 4-116.

Giroud, C.J.P., M. Saffron, A.V. Schally, J. Stachenko and E.H. Venning 1956 Production of aldosterone by rat adrenal glands in vitro. Proc. Soc. Exptl. Biol. Med. 92: 855-859.

Glagoleff, A.A., 1933 On the geometrical methods of quantitative mineralogic analysis of rocks. Tr. Inst. Econ. Min. and Metal. Moscow, 59: 1.

Greep, R.O. and I. Chester Jones, 1950 Steroid control of pituitary function. Recent Prog. Hormone Res. 5: 197-261.

Griffiths, K. and E.H. D. Cameron, 1970 Steroid biosynthetic pathways in the human adrenal. Adv. Steroid Biochem. Pharmacol. 2: 223-265.

Guillemin, R., G.W. Clayton, H.S. Lipscomb, and J.D. Smith, 1959 Fluorometric measurement of rat plasma and adrenal corticosterone concentration. J. Lab. Clin. Med. 53: 830-832.

Hennig, A., 1959 A critical survey of volume and surface measurements in microscopy. Zeis-Werkez. 30: 78-86.

Hirshfield, I.N. and S.B. Koritz, 1964 The stimulation of pregnenolone synthesis in the large particles from rat adrenals by some agents which cause mitochondrial swelling. Biochemistry (Washington) 3: 1994-1998.

Hoerr, N., 1931 The cells of the suprarenal cortex in the guinea pig. Their reaction to injury and their replacement. Amer. J. Anat. 48: 139-197.

Ichii, S., S. Kobayashi, N. Yago and S. Omata, 1967 Role of plasma cholesterol in rat adrenal corticosteroidogenesis in vitro. Endocrinol. Japon. 14: 138-142.

Idelman, S., 1966 Contribution a la cytophysiologie infrastructurale de la cortico-surrénale chez le rat albinos. Ann. Sci. Nat. Zool. 8 205-362.

Idelman, S., 1970 Ultrastructure of the mammalian adrenal cortex. Int. Rev. Cytol. 27: 181-281.

Jones, T. and K. Griffiths, 1968 Ultramicrochemical studies on the site of formation of dehydroepiandrosterone sulphate in the adrenal cortex of the guinea pig. J. Endocrinol. 42: 559-565.

Koritz, S.B., 1968 On the regulation of pregnenolone synthesis. In Functions of the adrenal cortex, Vol II (ed. K.W. McKerns). Appleton-Century-Crofts, New York. pp. 27-48.

Lever, J.D., 1952 Observations on the adrenal blood vessels in the rat. J. Anat. 86: 459-467.

Lever, J.D., 1954 Vascular zoning in the adrenal cortex of the normal and hypophysectomized rat with observations of the distribution of lipids. J. Endocrinol. 10: 133-146.

Long, J.A., 1973 The adrenal gland. In Histology (ed. R.O. Greep and L. Weiss). McGraw-Hill. New York. pp. 943-962.

Long, J.A., 1974 Zonation of the mammalian adrenal cortex. In Handbook of Physiology. Section 7, Endocrinology. The American Physiological Society, Washington, D.C. In press.

Loud, A.V., 1962 A method for the quantitative estimation of cytoplasmic structures. J. Cell Biol. 15 481-487.

Luft, J.H., 1961 Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409-414.

Luse, S., 1967 Fine structure of adrenal cortex. In The Adrenal Cortex. (ed. A.B. Eisenstein). Little, Brown and Co., Boston. pp. 1-59.

Magalhães, M.C. and M.M. Magalhães, 1969 A stereologic study of the effects of metopiron on the rat adrenal. Lab. Invest. 21: 491-496.

Magalhães, M.C. and M.M. Magalhães, 1972 Ultrastructural alterations produced in rat adrenal by aminoglutethimide. A stereologic and cytochemical study. Endocrinol. 90: 444-452.

Mäusle, E. and H. Scherrer, 1974 Ultramorphometric studies regarding the effect of sex-hormones on the adrenal cortex of the rat. Cell. Tiss. Res. 149: 113-120.

McKerns, K.W., 1968 Mechanisms of ACTH regulation of the adrenal cortex. In Functions of the Adrenal Cortex Vol. II. (ed. K.W. McKerns). Appleton-Century-Crofts, New York. pp. 479-537.

Mess, B. and L. Martini, 1968 The central nervous system and the secretion of anterior pituitary trophic hormones. In Recent Advances in Endocrinology (ed. V.H.J. James). Little, Brown and Co., Boston. pp. 1-49.

Mitchell, R.M., 1948 Histological changes and mitotic activity in the rat adrenal during postnatal development. Anat. Rec. 101: 161-185.

Morris, M.D. and I.L. Chaikoff, 1959 The origin of cholesterol in liver, small intestine, adrenal gland, and testis of the rat: dietary versus endogenous contributions. J. Biol. Chem. 234: 1095-1097.

Moses, H.L., W.W. Davis, A.S. Rosenthal and L.D. Garren, 1969 Adrenal cholesterol: localization by electron-microscope autoradiography. Science 163: 1203-1205.

Mostafapour, M.K. and T.T. Tchen, 1971 Evidence for another factor in the regulation of corticosterone biosynthesis by ACTH. Biochem. Biophys. Res. Commun. 44: 774-778.

Nishikawa, M., I. Murone, and T. Sato, 1963 Electron microscopic investigations of the adrenal cortex. Endocrinol. 72: 197-209.

Nussdorfer, G.G., 1970(a) The fine structure of the newborn rat adrenal cortex. Z. Zellforsch. 103: 382-397.

Nussdorfer, G.G., 1970(b) Effects of corticosteroid-hormones on the smooth endoplasmic reticulum of rat adrenocortical cells. Z. Zellforsch. 106: 143-154.

Nussdorfer, G.G. and G.M. Mazzocchi, 1972 A stereologic study of the effects of ACTH and cyclic 3', 5'-AMP on adrenocortical cells of intact and hypophysectomized rats. Lab. Invest. 26: 45-52.

Nussdorfer, G.G. and G.M. Mazzocchi, 1973 Effects of 3 ' , 5' cyclic nucleotides on adrenocortical cells of hypophysectomized rats. A stereologic and autoradiographic study. Lab. Invest. 28: 332-342.

Nussdorfer, G.G., G. Mazzocchi and L. Rebonato, 1971 Long-term trophic effect on ACTH on rat adrenocortical cells. An ultrastructural, morphometric and autoradiogrpahic study. Z. Zellforsch. 115: 30-45.

Nussdorfer, G.G., P. Rebuffat, G. Mazzocchi, A.S. Belloni, and V. Meneghelli, 1974 Investigations on adrenocortical mitochondria turnover I. Effect of chronic treatment with ACTH on the size and number of rat zona fasciculata mitochondria. Cell. Tiss. Res. 150: 79-94.

Palade, G.E., 1952 A study of fixation for electron microscopy. J. Exp. Med. 95: 285-297.

Palkovits, M. and J. Fisher, 1968 Karyometric Investigations. Budapest. Akademiai Kiado. pp. 347.

Perracchia, C. and B.S. Mittler, 1972 Fixation by means of glutaraldehyde-hydrogen peroxide reaction products. J. Cell. Biol. 53: 234-238.

Reiss, M., J. Balint, F. Oestreicher, and V. Aronson, 1936 Zur morphogenetischen wirkung und biologischen eichung der kortikotropen wirkstoffes. Endokrinologie 18:1-10.

Reiter, R.J. and D.J. Pizzarello, 1966 Radioautoradiographic study of cellular replacement in the adrenal cortex of male rats. Texas Repts. Biol. Med. 24: 189-194.

Rhodin, J.A.G., 1971 The ultrastructure of the adrenal cortex of the rat under normal and experimental conditions. J. Ultrastruct. Res. 34: 23-71.

Rosiwall, A., 1898 Uber geometrische gesteinsanalysen. Ein einfacher weg zur ziffernmässigen festellen des quantitätsverhaltnisses der mineralbestandteile gemengter gesteine. Verh. K.K. Geol. Reichsanst. 6: 143-175.

Sabatini, D.D., E.D.P. De Robertis and H.B. Bleichmar, 1962 Submicroscopic study of the pituitary action on the adrenocortex of the rat. Endocrinol. 70: 390-406.

Samuels, L.T. and T. Uchikawa, 1967 Biosynthesis of adrenal steroids. In The Adrenal Cortex (ed. A.B. Eisenstein). Little, Brown and Co., Boston pp. 61-102.

Scherle, W., 1970 A simple method for volumetry of organs in quantitative stereology. Mikroskopie, 26: 57-60.

Sekiyama, S., N. Yago, Y. Iwai, H. Kurokawa, F. Sato and A. Shiragai, 1971 A stereological description of size and number of zona fasciculata mitochondria in rat adrenal cortex after hypophysectomy. Endocrinol. Japon. 18: 365-369.

Sheppard, H., R. Swenson, and T.F. Mowles, 1963 Steroid biosynthesis by rat adrenal: functional zonation. Endocrinol. 73: 819-824.

Smith, P.E., 1930 Hypophysectomy and a replacement therapy in the rat. Am. J. Anat. 45: 205-273.

XUM

Swann, H.G., 1940 The pituitary-adrenocortical relationship. Physiol. Rev. 20: 493-521.

Tchen, T.T., 1968 Conversion of cholesterol to pregnenolone in the adrenal cortex: enzymology and regulation. In Functions of the Adrenal Cortex, Vol. II. (ed. K.W. McKerns). Appleton-Century-Crofts, New York. pp. 3-26.

Tomkeieff, S.I., 1945 Linear intersepts, areas and volumes. Nature (London) 155: 24.

Vasama, R., L. Kalliomaki, R. Vasama and E. Naatanen, 1957 The adrenal cortex and administration ACTH, cortisone and a combination of ACTH and cortisone. Ann. Med. Exp. Fenn. 35: suppl. 1, 1-8.

Venable, J.H. and R. Coggeshall, 1965 A simplified lead citrate stain for use in electron microscopy. J. Cell. Biol. 25: 407-408.

Walker, B.E. and E.G. Rennels, 1961 Adrenal cortical cell replacement in the mouse. Endocrinol. 68: 365-374.

Weibel, E.R., 1963 Principles and methods for the morphometric study of the lung and other organs. Lab. Invest. 12: 131-155.

Weibel, E.R., 1969 Stereological principles for morphometry in electron microscopic cytology. Int. Rev. Cytol. 26: 235-302.

Weibel, E.R., and R.P. Bolender, 1973 Stereological techniques for electron microscopic morphometry. In Principles and Techniques of Electron Microscopy (ed. M.A. Hayat). Vol. 3. Van Nostrand Reinhold Co., New York. pp. 237-296.

XUM

Weibel, E.R., G.S. Kistler and W.F. Scherle, 1966 Practical stereological methods for morphometric cytology. J. Cell Biol. 30: 23-38.

Wright, N.A., 1971 Cell proliferation in the prepubertal male rat adrenal cortex: an autoradiographic study. J. Endocrinol. 49: 599-609.

Yago, N., S. Sekiyama, H. Kurokawa, Y. Iwai, F. Sato and A. Shiragai, 1971 Volume increase in rat adrenocortical mitochondria by single ACTH administration. A stereological study. Endocrinol. Japon. 18: 359-364.

Yoshimura, F., K. Harumiya, N. Suzuki, and S. Totsuka, 1968 Light and electron microscopic studies on the zonation of the adrenal cortex in albino rats. Endocrinol. Japon. 15: 20-52.

APPENDIX

Method for Determining Corticosterone Plasma Levels

This fluorometric method is a modification of the one developed by Guillemin et al., (1959).

Plasma, 0.5 ml sample:

1. Partition and Extraction. 0.5 ml of plasma is placed in a 15 ml centrifuge tube. The volume is brought to 2.0 ml with distilled water. Add 4.0 ml isooctane; mix with a vortex stirrer for 15 seconds and then centrifuge fo 3-4 minutes. Aspirate off isooactane layer and discard. Add 2.0 ml distilled water followed by 5.0 ml chloroform. Mix for 60 seconds, and a thick emulsion forms. Centrifuge for 10 minutes; aspirate off top aqueous layer and discard.
2. NaOH Washing. Add 0.5 ml O.IN NaOH. Shake gently for 15 seconds and freeze in dry ice to facilitate removal of emulsion. Thaw and gently invert tube to further remove emulsion. Centrifuge for 3-4 minutes.
3. Extraction in H_2SO_4. Transfer 4.0 ml of the chloroform extract (bottom layer) to another 15 ml centrifuge tube. Add 0.6 ml of absolute ethanol and 1.4 ml 30 N H_2SO_4 to make up 2 ml of solution (a ratio of 3:7 volume by volume). Note time here. Mix for 15 seconds. Aspirate off chloroform (top layer) and discard.
4. Fluorometry. About 1.0 ml of the acid extract is transferred to fluorometer tubes. Read 60 minutes after adding H_2SO_4. A Turner fluorometer was used.

5. Standard. 0.75 ml of a stock solution containing 2.0 μg corticosterone per ml in absolute ethanol is placed in a 50 ml centrifuge tube and carefully evaporated to dryness by a stream of air. Add 2.0 ml of distilled water and run through the whole procedure along with the unknowns.